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**The genetic diversity of *Trypanosoma cruzi*:  
multiclonality of natural populations, and  
characterisation of Paraguayan isolates and  
experimentally derived *T. cruzi* I hybrids**

A thesis submitted in fulfilment of the requirements for the  
degree of Doctor of Philosophy of the University of London

**June 2003**

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## Abstract

The aims of this PhD programme were: optimisation of conditions for clonal isolation of *Trypanosoma cruzi* from individual triatomine bugs and mammals; molecular characterisation of clonal isolates from field sites in Paraguay, with special consideration given to host/vector associations and transmission cycles, and further characterisation of putative parents and experimental hybrid progeny of *T. cruzi* 1, with comparison of hybrid genotypes and field isolates from Paraguay.

The development of an optimised solid medium plating technique allowed the resolution of mixed infections by the isolation of clonal colonies of *T. cruzi* from single triatomine bugs and experimentally infected mice. Some nutrient plate types produced rapid growth with high plating efficiencies and longer colony viability in comparison with previous methods. Using this technique growth was also possible without the use of a CO<sub>2</sub> incubator.

Paraguayan isolates were characterised from mammal hosts, sylvatic *T. infestans*, and domestic *T. infestans* from several geographical locations. Isolates were characterised by phenotyping and by genotyping large and small subunit rRNA and mini-exon genes. Analysis enabled assignment of isolates into one of two major lineages (TC1 or TC2), and further attribution to one of five subgroups of TC2 (a-e). In Paraguay isolates were all subgroups of TC2. We identified TC2b, TC2c, TC2d and TC2e circulating in Paraguay. TC2b, TC2d and TC2e were present in domestic cycles (with a predominance of TC2e, 55.2%) and TC2c, TC2b and TC2d in sylvatic cycles. Two species of armadillo (*Dasypus novemcinctus*; TC2c, TC2d and *Euphractus sexcinctus*; TC2b, TC2d) were identified as sylvatic reservoirs of *T. cruzi*. These findings accord with our theory that terrestrial armadillos and rodents may have an evolutionary association with TC2.

Sylvatic colonies of the vector *T. infestans* were identified from two locations in the Paraguayan Chaco. *T. cruzi* infection in sylvatic *T. infestans* was identified as TC2b. This vector was previously regarded as almost exclusively domestic, and this finding has significant implications for future vector surveillance programmes in Paraguay.

By further characterisation of hybrid clones it has been proved for the first time that *T. cruzi* has an extant capacity for genetic exchange. Two genetically transformed

putative parental types and six double drug resistant clones, previously isolated by co-infection of mammal cells, were analysed by isoenzyme phenotype, random amplified polymorphic DNA (RAPDs), karyotype analysis and DNA sequencing. These results, together with parallel work performed within our group, suggest an unusual mechanism of genetic exchange involving fusion of parental genotypes and subsequent loss of alleles in polyploid progeny, in conjunction with homologous recombination, and uniparental inheritance of kinetoplast maxicircle DNA. There were parallels between experimentally generated hybrids and natural isolates. The implications of genome fusion and aneuploidy are profound and the mechanism is likely to be widespread providing a possible means of rapid speciation and evolution.

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## **List of abbreviations**

6PGD - 6-phosphogluconate dehydrogenase (decarboxylating)  
ALAT - alanine aminotransferase  
ASAT - aspartate aminotransferase  
bp - base pair  
CD3, 4, 8 - Clusters of differentiation  
GM-CSF – granulocyte macrophage colony stimulating factor  
IgG - Immunoglobulin G  
IgE – Immunoglobulin E  
IgM – Immunoglobulin M  
IL-2 – Interleukin 2  
DALY – disability adjusted life year  
ELISA - enzyme-linked immunosorbent assay  
EtBr - ethidium bromide  
G6PD – glucose 6-phosphate dehydrogenase  
GPI - glycosyl-phosphatidylinositol or glucose phosphate isomerase  
HEPES - N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic] acid, sodium salt  
ICD - isocitrate dehydrogenase  
IEA - isoenzyme electrophoresis  
IgG - immunoglobulin G  
kb - kilobase pairs  
kDa - Kilodalton  
LDH - lactate dehydrogenase  
LSHTM - London School of Hygiene and Tropical Medicine  
ME - malic enzyme  
min - minutes  
MPI - mannose phosphate isomerase  
mRNA - messenger RNA  
Mya - million years ago  
Mybp – million years before present  
NAD -  $\beta$ -nicotinamide adenine dinucleotide  
NADH -  $\beta$ -nicotinamide adenine dinucleotide, reduced form

NADP -  $\beta$ -nicotinamide adenine dinucleotide phosphate  
PBS - phosphate buffered saline  
PCR - polymerase chain reaction  
PFGE - pulse-field gradient gel electrophoresis  
PGM - phosphoglucomutase  
RAPD - random amplified (amplification of) polymorphic DNA  
RFLP - restriction fragment length polymorphism  
rRNA - ribosomal RNA  
SDS - sodium dodecyl sulphate  
SSC - sodium citrate buffer  
TNF – Tumor necrosis factor  
USA - United States of America  
UV - ultra-violet  
v/v - volume/volume  
w/v - weight/volume  
WHO - World Health Organisation

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## 1.0 INTRODUCTION

### 1.1 Classification, biology and life cycle of *Trypanosoma cruzi*

Organisms within the genus *Trypanosoma* (Kinetoplastida: *Trypanosomatidae*) are digenetic unicellular parasites. *T. cruzi*, *Leishmania* and the African trypanosomes, are all members of the family *Trypanosomatidae*. They are characterised by a bar-shaped kinetoplast consisting of tightly coiled circles of DNA in the terminal region of the organism's single large mitochondrion. Trypanosomes possess a single flagellum originating from the flagellar pocket which is adjacent to the kinetoplast in all stages of the trypanosome life cycle. Three main morphological forms exist in the *T. cruzi* life cycle (Figure 1)

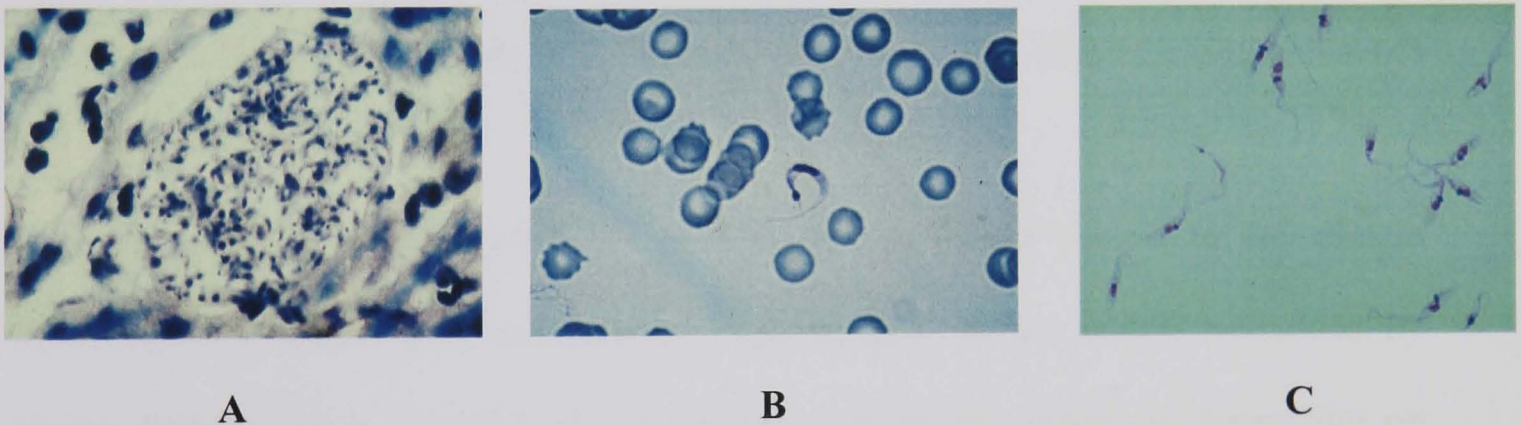


Figure 1 - A. Intracellular amastigote, B. mammalian bloodform trypomastigotes, C. epimastigote forms.

The amastigote form multiplies in mammalian phagocytic and non-phagocytic cells by binary fission. The epimastigote form divides by binary fission in the hindgut of the triatomine vector. Trypomastigotes, the non-replicating infective stage, are found in the rectum of the triatomine vector and excreted with infected bug faeces. These are often referred to as infective metacyclics. Mammalian trypomastigotes are found in the circulatory system of the infected host. All three forms can be distinguished morphologically (Hoare, 1972). Infective metacyclics are discharged with bug faeces (Figure 2) during or following a blood meal.

Parasites enter the host through the bite wound, the conjunctiva, or through mucous membranes. Following entry into the host, trypomastigotes penetrate phagocytic or non-phagocytic cells. The method of entry into host cells is uncertain but involves disruption of a  $\text{Ca}^{2+}$  regulated lysosomal exocytic pathway (Sibley & Andrews, 2000). Within the cell, a membrane bound vacuole containing *T. cruzi* fuses with lysosomes to form a phagolysosome. The organism escapes into the cytosol and differentiates into a round non-flagellated amastigote, dividing by binary fission to produce a pseudocyst. Some amastigotes differentiate into trypomastigotes prior to cell lysis, releasing up to 500 motile, non-replicating parasites into interstitial fluids and the circulatory system. Circulating trypomastigotes can infect a variety of mammalian cells, however high parasite burdens are most commonly found in reticuloendothelial cells of the spleen, liver, lymphatics and also in cardiac, smooth and skeletal muscle.

Circulating trypomastigotes are ingested by the insect vector. In the midgut differentiation occurs into non-infective epimastigote forms which reproduce by binary fission. After a period of 3 to 4 weeks following infection, depending on parasite strain and microecological factors, epimastigotes transform into infective metacyclic trypomastigotes. These are excreted during or after a blood meal to continue the cycle. Host infection is also possible through blood transfusion (Schmunis, 1999), organ transplant, (Riarte *et al.*, 1999) oral ingestion, laboratory accidents and congenital transmission (Nisidia *et al.*, 1999).



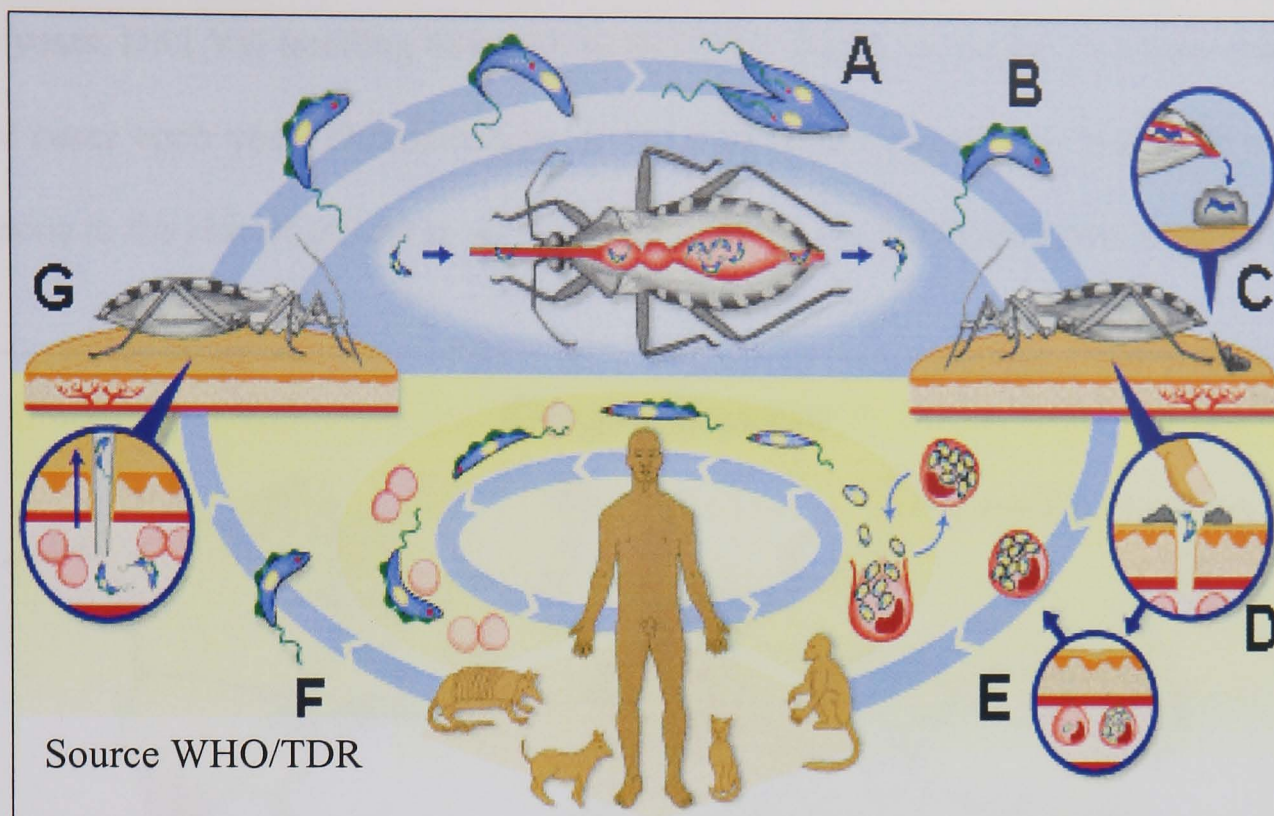


Figure 2 - Summary of the life cycle of *Trypanosoma cruzi*. A. Dividing epimastigotes, B. Infective metacyclics, C. Excreted infective metacyclics, D. Host cell infection, E. Pseudocysts containing dividing amastigotes, F. Circulating trypomastigotes, G. Ingestion of trypomastigotes by insect vector.

## 1.2 Epidemiology, distribution and public health importance.

*T. cruzi* is widespread throughout the Americas mainly among small wild mammals (enzootic sylvatic cycle). Human Chagas disease constitutes a more recent situation, in which bio-ecological and socioeconomic factors leave rural poor populations of South and Central America in contact with the sylvatic cycle, where the parasite is transmitted by natural vectors of the infection (Coura *et al.*, 1999, Dias-Lima & Sherlock, 2000). From the public health standpoint, the importance of Chagas disease remains largely correlated to the resultant "domestic" cycle, in which triatomine bugs colonise houses, and against which most control measures are directed.

*T. cruzi* can be detected over a wide area of America, from latitude 42 N to latitude 46 S, (Figure 3) although the distribution of wild vectors and reservoirs is much greater than that of the human disease. Chagas disease is considered to be the most important parasitic infection in Latin America (in terms of disability adjusted



life years, DALYs) totalling 680,000, with 21,000 deaths annually. There are 300,000 new cases each year. Domiciliation of the triatomines exposes at least 120 million persons to the risk of infection, in 18 countries from south of the United States of

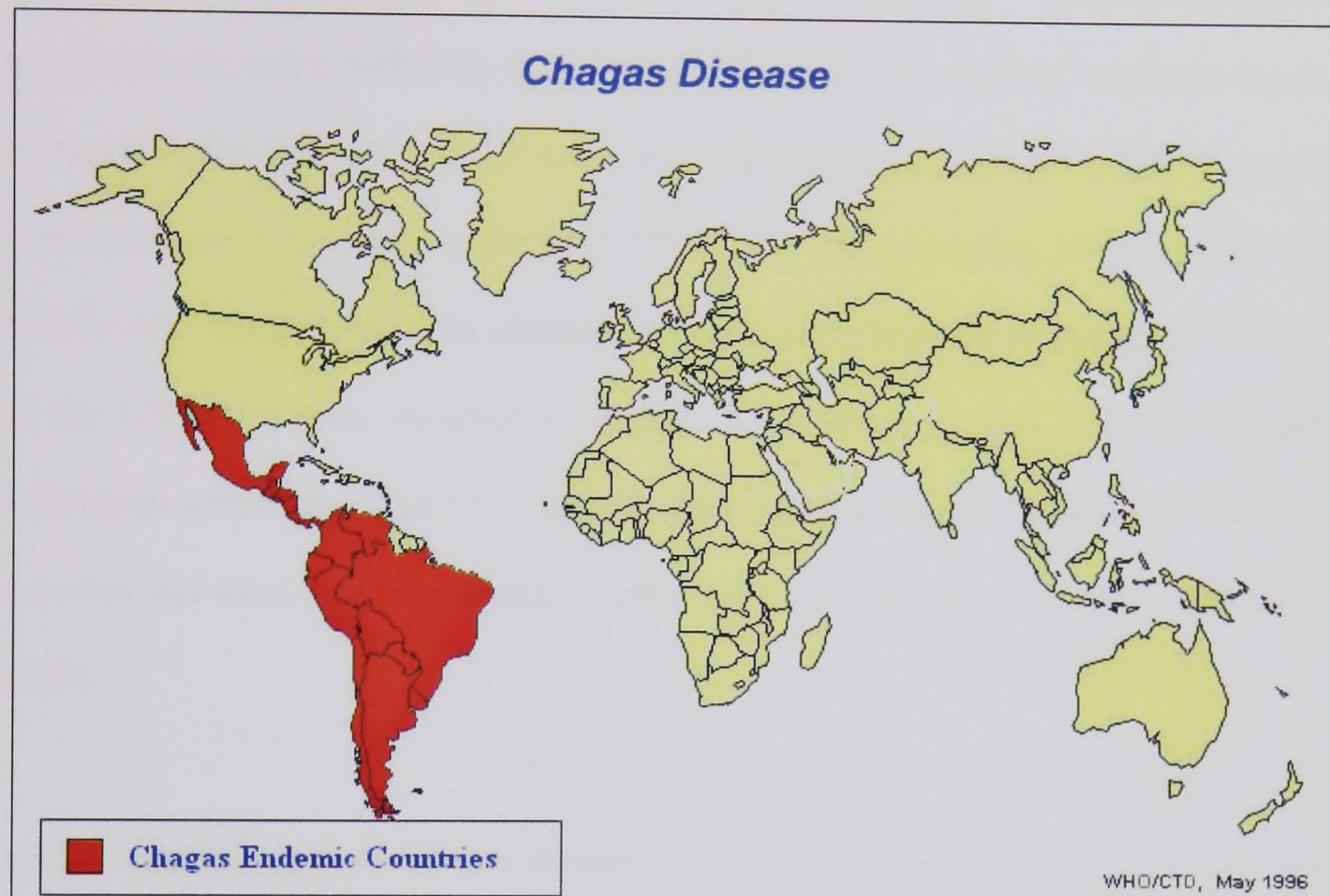


Figure 3 – Distribution of Chagas disease.

America to the province of Chubut, Argentina (Moncayo, 1992). This comprises almost 25% of the population of Latin America. Better socioeconomic conditions and behaviour of the local species of triatomines make vectorial transmission extremely rare in the United States, although transfusional transmission via immigrant blood donors represents a significant risk (Shulman, 1999). In endemic countries, it is estimated that 11 million people are infected by the parasite (WHO 2002). Chagas disease is not an isolated problem among Latin American populations. In endemic areas it is closely associated with typical "social" diseases such as malnutrition, diarrhoea, tuberculosis and other parasitic diseases (Andrade *et al.*, 1995). In the past

Chagas disease was considered to be a typical rural disease of Latin America. However the trend towards urbanisation is modifying the spectrum of human American trypanosomiasis across the continent (Guhl *et al.*, 2000).

Recent economic and social changes are stimulating rural to urban migration in many endemic areas, with more than 60% of the population presently settled in urban centres in Latin America. It is estimated that, due to migration, about 300,000 infected individuals are living today in the city of Sao Paulo with more than 200,000 in Rio de Janeiro and Buenos Aires. In addition, infected individuals are migrating northward to the USA and even eastward to Europe. It is estimated that 100,000 infected individuals are living in the USA, most of them immigrants from Mexico and Central America and these present a further transfusional risk (Kerndt *et al.*, 1991; Shulman, 1999).

### **1.3 Economic impact of Chagas disease**

The economic impacts of Chagas disease are enormous (WHO, 2002). It is estimated that 752,000 working years per year are lost due premature deaths corresponding to 1208.5 US\$ million/year. In Brazil, at least 10% of infected patients develop severe chronic cardiac or digestive involvement, the medical costs for obligatory treatment throughout their lifetime is an estimated US\$53 million. In addition chronic absenteeism of 75,000 Brazilian chagasic workers with serious cardiac damage could represent minimum losses of US\$56 million (WHO, 1991; Schofield & Dias 1999). In Latin America as a whole economic loss due to early disability amounted to US\$8156 million, which was equivalent to 2.5 percent of the external debt of the continent in 1995 (WHO, <http://who.int/ctd/chagas/disease.htm>).

The predicted cost for effective implementation of the southern cone project (1991-2000) was between US\$190 million and US\$350 million. By 2001 the southern cone countries had contributed US\$320 million (Dias *et al.*, 2002). Brazil alone has invested US\$420 million between 1975-1995. This has been offset by reduced mortality and medical savings (US\$53 million). Rates of return of investments are impressive. Within the southern cone countries return is 14% (Schofield & Dias, 1991). Specific examples include 30% for Brazil (Akhavan, 2000), and 64% for Argentina (Basombrio *et al.*, 1998).

#### **1.4 Clinical aspects**

Clinical infection comprises three stages and is responsible for an estimated 10% mortality in infected individuals (Chapadeiro, 1999). An initial acute phase is accompanied by variable symptoms which typically become apparent two weeks or more following infection. These include fatigue, fever, facial swelling or swelling at the point of entry (chagoma). The acute phase may last for up to four weeks with an associated mortality of 2-7%. Children under the age of five and immunodeficient patients represent the highest risk of mortality (Ferreira *et al.*, 1996). An intermediate stage follows acute infection, there are no symptoms, this stage may last for many years. During the intermediate phase no trypomastigotes are detectable in the bloodstream by direct microscopy, but they are present in low numbers for life, as is antibody (IgG; immunoglobulin G) against *T. cruzi* (Miles, 1998).

One third of those infected will go on to develop the chronic phase of the disease within 10 to 30 years. Infection results in destruction of the ganglia of the cardiac and digestive tract leading to chronic phase megasyndromes (megaoesophagus and megacolon) the precise pathogenesis of which is poorly understood.

Chagas disease transmission via blood transfusion is significant risk factor throughout Latin America. Since the 1950s, many scientific publications have shown that the transmission of *T. cruzi* by blood transfusion from infected donors is frequent in some endemic areas (WHO, 2002; Miles, 1998; Dias & Schofield, 2002).

Natural, non vector borne transmission of the parasite involving congenital and oral routes can also occur in both sylvatic and domestic cycles, while transfusional transmission is an artificial mechanism depending on socio-epidemiological situations and directly linked with the quality of the health system in endemic countries (Schmunis, 1999; Schmunis *et al.*, 2001; WHO, 2002).

## **1.5 Immune response and pathogenesis**

### **1.5.1 Avoidance of the immune response**

The mechanisms by which *T. cruzi* avoids the immune response are far from understood. Avoidance of the immune system is particularly important during the acute phase (Hayes & Kierzenbaum, 1981) when cell surface receptors on the parasite avoid eliciting an immune response. Nonspecific humoral and cellular immune suppression by *T. cruzi* antigens was demonstrated by Tarleton and Scott (1987). Similarly parasites have been found to excrete products which inhibit CD3, CD4, CD8 and IL-2 receptors on human lymphocytes, resulting in a possible depression of the immune response (Sztein *et al.*, 1990). Norris & Schrimpf (1994) reported on the binding of the third component of complement by a specific trypomastigote surface glycoprotein inhibiting activation of the alternative complement pathway and restricting activation of the classical pathway. A second trypomastigote protein, between 87-93kDa in size also inhibits the formation of the alternative pathway C3 convertase (Joiner *et al.*, 1998). In addition trypomastigotes have been shown to

demonstrate resistance to the lytic effects of complement (Krautz *et al.*, 2000). Negatively charged mucin like molecules have been identified on the parasite surface and protect the organism from anti-alpha galactosyl antibodies (Pereira-Chioccola *et al.*, 2000). If mucins are removed, *in vitro* by  $\text{MgCl}_2$ , trypomastigote forms become susceptible to antibody.

### 1.5.2 Immune response

Mechanisms of host resistance to infection by *T. cruzi* have yet to be fully elucidated. Infection invokes a cellular and humoral immune response. *T. cruzi* specific antibodies can typically be detected within 2 weeks, of infection, seropositivity lasting throughout life. All immunoglobulin subclasses are elevated (Miles, 1998), the subsequent hypergammaglobulinaemia resulting in increased levels in IgG1, IgG2a, IgG2b and IgM which are present throughout infection. Elevated levels of IgE, IgM and IgG2a persist for up to 13 weeks after which levels of IgG2a and IgM fall significantly. During the acute phase of infection the production of cytokine interleukin-2 (IL-2) is depressed increasing survival of infective trypomastigotes (Soong & Tarleton, 1994).

Following acute infection an immune response is mounted which can suppress but not eradicate infection. Both  $\text{CD4}^+$  and  $\text{CD8}^+$  cells have a role in immunity (Rottenberg *et al.*, 1993). Cytotoxic T cells have the ability to recognise and damage infected cells exposing the intracellular amastigotes to cytolytic attack. Similarly, inflammatory type cells with and without activating and enhancing cytokines and FC-receptor bound antibodies have been shown to kill mammalian forms of *T. cruzi* (Kierszenbaum *et al.*, 1994). The killing of parasites observed in macrophages depends upon the production of interferon-gamma, synergistic with TNF-alpha,



triggering macrophage activation and nitric oxide production (Munoz-Fernandez & Frenso, 1992; Silva *et al.*, 1995).

The role of cytokines and antibodies has been extensively studied. In the acute phase IFN-gamma, TNF-alpha, IL-12, and GM-CSF have been associated with resistance, in contrast the presence of IgF-SS and IL-10 have been associated with susceptibility (Umekita & Mota, 2000). More recently it has been shown that antibodies produced in the chronic stage of infection also render trypomastigotes sensitive to immune responses (Krautz *et al.*, 2000). The suppression of parasitaemia depends on the ability to remove trypomastigotes.

### **1.5.3 Pathogenesis**

Mechanisms of pathology, that produce chagasic cardiac and digestive disease, are not fully understood. Initial theories related to the direct destruction of infected tissue by high parasitemia, particularly in the acute phase of infection. Teixeira *et al.* (1995) reported amastigote nests in adrenal glands and central veins in 50% of infected patients. Immunohistological staining of cardiac muscle isolated from patients with chronic chagasic myocarditis has revealed *T. cruzi* antigens indicating the existence of residual pseudocysts (Guarner *et al.*, 2001). It has been suggested that the disease is primarily a neuropathy which results in intestinal and cardiac enlargement by the destruction of ganglionic nerve fibres. The proposed mechanism is the destruction of vagal neurones by the parasite, resulting in parasympathetic abnormalities and an autonomic imbalance (sympathetic dominance) leading to progressive dilatation and eventual organ failure (Davila *et al.*, 1998). However evidence also points to an autoimmune response playing a role in pathology, particularly as parasite densities are low in chronically infected patients. Using mouse

models Hudson (1983) demonstrated that antigens from *T. cruzi* illicit an inflammatory response. However no correlation was demonstrated between the intensity of lesions and quantity of antigen produced, indicating an autoimmune component in the host response.

The major *T. cruzi* antigen cruzipain has also been shown to induce a powerful autoimmune response. Cruzipain triggers immunoglobulin G antibody binding to a 210kDa antigen from skeletal muscle. Subsequent histological studies and increased creatine kinase activity provide strong evidence for an anti-cruzipain immune response leading to tissue damage (Giordanengo *et al.*, 2000). In chronic chagasic cardiomyopathy evidence also indicates a T cell-dependent autoimmune response (Silva-Barbosa & Savino, 2000). Pathogenesis is thus likely a combination of multiple factors, possible outcomes include myocarditis, immunodepression, severe fibrosis, microvessel dilatation and organ failure with the initial process triggered by the direct presence of the parasite (Soares *et al.*, 2001).

## **1.6 Treatment**

Prompt treatment for those infected with *T. cruzi* is imperative. Current therapies have a small therapeutic window and are most successful during acute phase infection with a success rate of approximately 60%. The two effective therapies are nifurtimox (Lampit, a nitrofurantoin derivative) and benznidazole (Radanil, a nitroimidazole derivative). Nifurtimox induces oxidative stress, but also interferes with glucose 6-phosphate dehydrogenase often causing severe adverse effects necessitating hospitalisation. The treatment exhibits intracellular activity against amastigotes as well as activity against trypomastigotes. Patient dosages are 10mg/kg for adults and 15mg/kg for infants over a 60-90 day period (Miles, 1998).

*T. cruzi* heterogeneity and variations in drug susceptibility are increasingly recognised as an important consideration. Murta *et al.*, (1998) identified strains, analysed using different molecular markers, that were naturally resistant and susceptible to benznidazole and nifurtimox. This revealed one strain containing an heterozygous profile (named zymodeme B) was highly sensitive to chemotherapeutic agents and occurred predominantly in areas where treatment had previously been reported to be more effective.

Intricate surgical intervention techniques have been developed for the treatment of mega syndromes (da Silva, 1999). The implantation of pacemakers has aided patients suffering from arrhythmias. Verapamil, a calcium channel blocker has been demonstrated to reduce mortality in animal models (Chandra *et al.*, 2002).

## **1.7 Control of Chagas disease**

### **1.7.1 Transfusional control**

In the 1970s to 1980s Latin America experienced a major shift in socio-economic demography resulting in rural migration towards cities. This had profound epidemiological consequences for Chagas disease. Transmission from unscreened blood stocks became a major risk (Wendel, 1998; Schmunis, 1999). Transfusional infection was suspected in 1935, but it was not until the AIDS epidemic in the 1980s that effective blood screening programmes began. During this period prevalence in blood stocks ranged from 0.15% in Ecuador to 40% in Bolivia (Dias *et al.*, 2002). Venezuela (4%), Paraguay, Chile, Argentina and Guatemala (8%) have also reported significant contamination of blood stocks. This was far higher than transfusional risk of hepatitis and HIV. The strategy for control of transfusional transmission is detection of suspect blood and chemoprophylaxis using trypanocidal drugs (WHO).

2002; Wendel, 1998). Screening is an integral function of national vector control programmes.

1.7.2 Vector control

There are currently three major control initiatives focusing on the eradication of vectorial and transfusional transmission in South America. Control is focused on the eradication of domestic bug populations. *T. cruzi* itself is a zoonosis with a large number of sylvatic reservoirs, so eradication is not possible.

The most successful programme is the southern cone initiative (Figure 4). Established in 1991 and involving of the so called 'southern cone countries' (Schofield

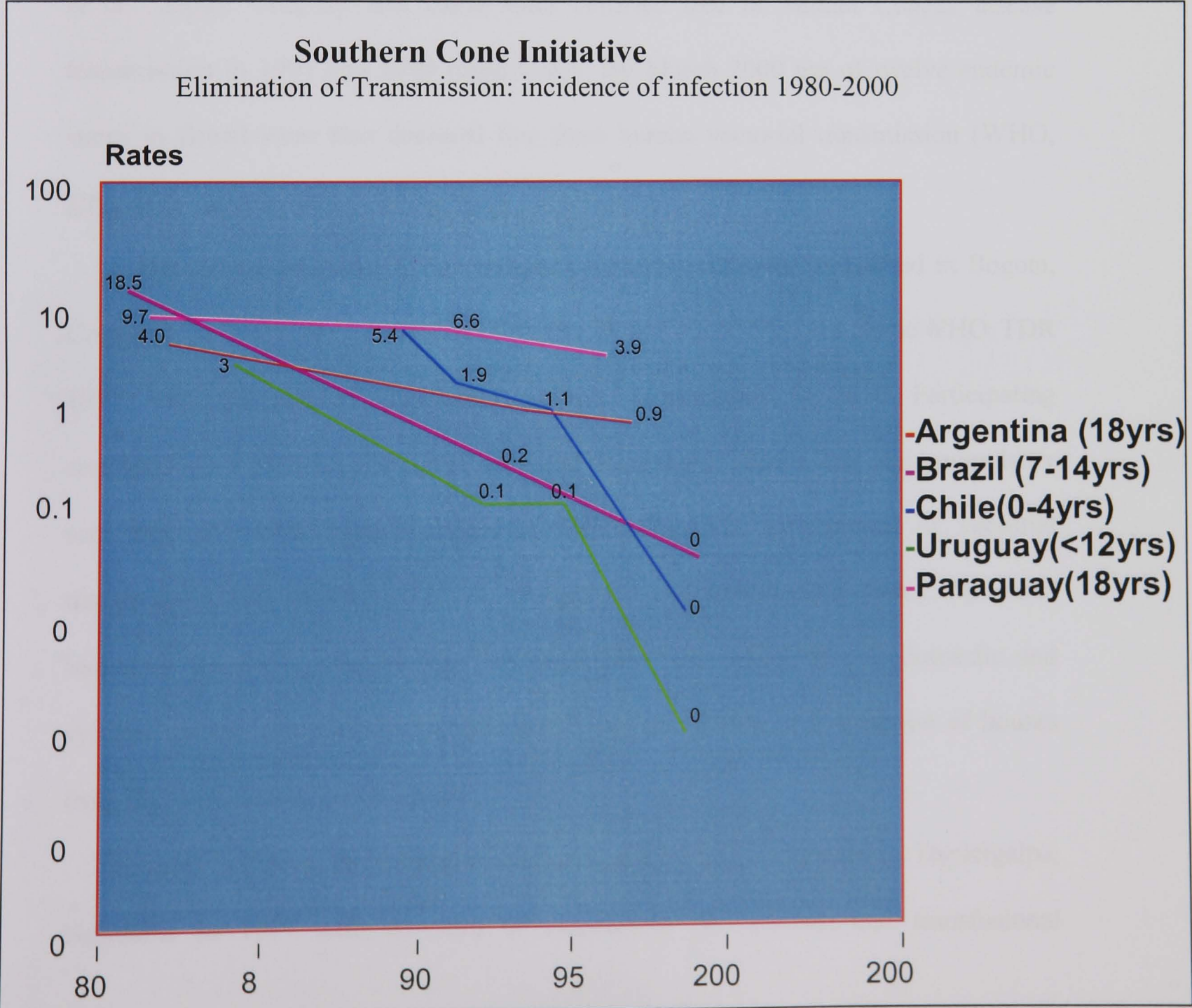


Figure 4 – Incidence of infection 1980 - 1999

& Dias, 1999), this brought together an unprecedented amalgamation of resources to reach predefined goals. Initial aims (Phase I) was the compulsory screening of all donor blood, and is currently operative in all participating countries. The second goal (Phase II) is the interruption of vectorial transmission, due to *Triatoma infestans*, using a formalised spraying campaign, within a specified period. Participating countries, and proposed eradication dates, were Uruguay, 1997; Chile, 1999; Brazil, 2000; Argentina, 2003; Bolivia 2005, and Paraguay, 2005. Elimination in southern cone countries would reduce infection rates by 70% in the whole of Latin America due to concentration of the affected population. To date the programme has been largely successful with vectorial and transfusional transmission reduced by 72% (Dias *et al.*, 2002). Uruguay and Chile were certified free of human Chagas disease transmission in 1997 and 1999 respectively. By March 2000 ten of twelve endemic states in Brazil were also declared free from human vectorial transmission (WHO, <http://who.int/ctd/chagas/disease.htm>).

The second initiative is the Andean Countries initiative, formalised in Bogota, Colombia in 1997 and consolidated by the WHO (UNDP/World Bank/WHO TDR 1997) with the aim of eliminating vectorial transmission by 2010. Participating countries are Colombia, Ecuador, Peru and Venezuela. Phase one, the compulsory screening of blood, was launched in 1993, Phase II, interruption of vectorial transmission, was launched in 1997. The presence of vectors other than *T. infestans*, including *Rhodnius prolixus* and *Triatoma dimidiata*, which live in domestic and sylvatic cycles, complicates control efforts, due to the risk of re-invasion of houses from the surrounding environment.

The third initiative, the Central American initiative was formalised in Tegucigalpa, Honduras in 1997 with the aim of elimination of vectorial and transfusional

transmission by 2010. Participating countries are Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama. Highest prevalence of vectors (*R. prolixus*, *T. dimidiata* and *Rhodnius pallescens*) is in El. Salvador. A major challenge in this initiative is that the primary vectors of *T. cruzi* are considered not restricted to domestic habitats. Re-invasion by infected bugs from the surrounding environment requires a sustained long term vigilance programme (Ponce, 1999, Dias *et al.*, 2002).

### **1.7.3 Campaign strategies**

Vector control programmes in member countries tend to be based on those successfully implemented in the Brazilian model. This required an area of over 3 million km<sup>2</sup>, covering 2400 municipalities to be treated, with 25 million people at risk (Dias, 1984). The Brazilian programme follows an integrated approach implemented by SUCAM (Superintendencia de Campanhas de Saude Publica) operating in all states except Sao Paulo which operates under a separate body known as SUCEN (Superintendencia de Controle de Endemias). The programme has been a huge success with a virtual interruption of domestic and vectorial transmission although surveillance is vital for continued success (Silveira & Vinhaes, 1999).

There are three separate components in the Brazilian control programme (Costa *et al.*, 1998). An initial preparatory phase, during which areas are mapped, all houses and outbuildings searched manually with a flashlight, forceps and irritant pyrethroid spray. The objective is to accumulate distribution and prevalence data, assess costs and assemble resources. The second stage is the attack phase during which all properties and outbuildings are sprayed irrespective of the presence triatomine populations. This process is repeated within 3-6 months if the number of infected houses in the municipality is above 5%. The third and final phase is the vigilance



phase. Buildings are periodically surveyed using public health officials, members of the local community, health education and passive detection methods. Residences found to be positive are sprayed immediately.

#### **1.7.4 Unresolved control issues**

Perhaps the primary issue regarding continued control success, is the need for sustained surveillance and selective intervention. This in turn is dependent upon political will. Decentralisation of control programmes has frequently been cited as a major concern due to lack of resources, limited infrastructure, and lack of expertise at local levels (Dias *et al.*, 2002). A reduction in surveillance will, in all probability, result in re-establishment of triatomine vectors. At the time of writing the declining economic status of some Latin countries (particularly Argentina, Brazil, Ecuador, and Venezuela) may result in a decline of prioritised investment in Chagas control.

There remain three main geographical areas of concern. Control of Chagas disease is minimal in the vast Amazon basin, Mexico and the USA. In the Amazon human colonisation in frontier towns and the change in land use represents a relatively recent threat. In the Brazilian Amazon alone there have been 300 Chagas cases within the last 10 years (Dias *et al.*, 2002). Domiciliation of previously silvatic vector species has also been reported (Coura *et al.*, 2002). In the USA the primary threat comes from transfusional infection by inadequately screened bloodstocks. Similarly, Mexico presents a worrying case with low rates of blood screening and the presence of vector-borne transmission. It is estimated that over one million individuals may be infected (Guzman-Bracho, 2001).

1.8 Host range of trypanosomes

Trypanosomatid flagellates have the broadest host range of any parasitic eukaryotes (Vickerman, 1994). They have been identified in the blood, other body fluids and tissues of all vertebrate classes. *Leishmania* spp primarily parasitise mononuclear phagocytes of lizards and mammals (Figure 5). Other related genera

Taxonomy of Kinetoplastid Protozoa			
Order	Suborder	Family	Genus
Kinetoplastida	Bodonina	Bodonidae	Bodo Ichtyobodo
		Cryptobiidae	Cryptobia Trypanoplasma
	Trypanosomatina	Trypanosomatidae	Trypanosoma Leishmania Endotrypanum Crithidia Blastocrithidia Leptomonas Herpetomonas Phytomonas

Source - [www.hhmi.ucla.edu](http://www.hhmi.ucla.edu)

Figure 5 – Taxonomy of the Kinetoplastida. The Kinetoplastids and euglenoids belong to the phylum – Euglenozoa.

include *Crithidia*, *Blastocrithidia*, *Herpetomonas*, *Rhynchoidomonas* and *Proteomonas* (Vickerman, 1994) are usually single host gut parasites associated with a wide range of insects. An enormous range of trypanosomatids have been identified in the latex and phloem of flowering plants. Despite the apparent success in utilising a wide range of ecological niches there are noticeable absences. To date no free living trypanosomes have been identified. Additionally with the exception of arthropods, for



reasons unknown, other invertebrates, and non flowering plants do not appear to harbour trypanosomes (Podlipaev, 2000). Those with vertebrate hosts involve cyclical development involving a leech or arthropod vector.

### 1.9 Vertebrate hosts of *T. cruzi*

Over 150 mammal species comprising 24 families, are recorded as infected with *T. cruzi* or *T. cruzi*-like trypanosomes. Prevalence rates for sylvatic vertebrate hosts



Figure 6 – *Dasypus novemcinctus*, a sylvatic reservoir of *T. cruzi*.

have seldom been determined accurately.

Nevertheless it is clear that some mammal species, such as the marsupial *Didelphis marsupialis* and *Philander opossum*, and the

edentate *Dasypus novemcinctus* (nine banded

armadillo, Figure 6) often have a high

prevalence of infection. Major domestic

reservoirs include dogs (Castanera, 1998), guinea pigs, rats and mice (Valente *et al.*,

1999; Fujita *et al.*, 1994). Cattle and livestock usually have low infection rates,

although high numbers of bugs may be frequently found in animal pens. Chickens,

although not susceptible to infection, have epidemiological significance, as they can

often sustain significant numbers of triatomine bugs (Lopez *et al.*, 1999).

The resolution of host vector associations has important implications for epidemiology and control of Chagas disease, particularly with respect to vectorial re-invasion from sylvatic environments (Dias-Lima & Sherlock, 2000) and the identification of reservoirs of *T. cruzi* (Jansen *et al.*, 1999).



## 1.10 Host / vector associations.

### 1.10.1 Triatomine vectors

The haematophagous insect vectors of Chagas disease belong to a subfamily of the Reduviidae, of the order Hemiptera, suborder Heteroptera. The Triatominae are

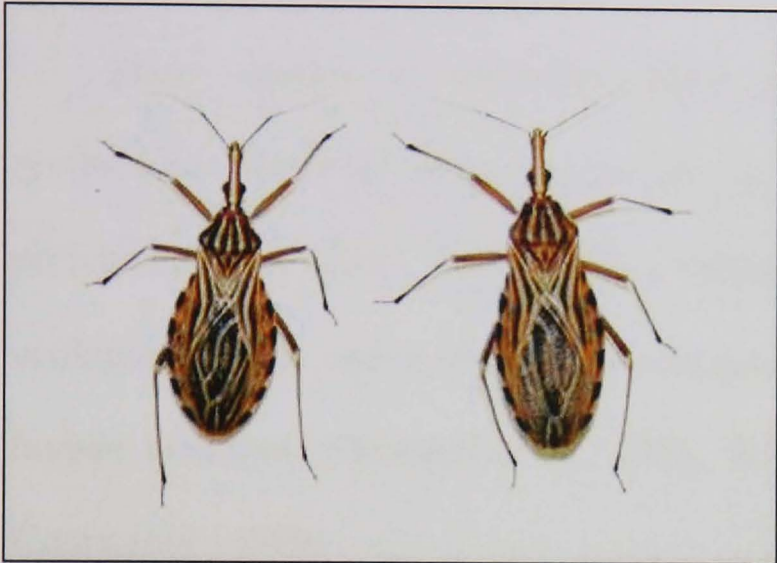


Figure 7 – *Rhodnius prolixus*, male (right), female (left). This vector is a major vector of *T. cruzi* in northern countries of South and Central America.

segregated into five tribes. Over 130 species and 17 genera of Triatominae have been described based on morphological characteristics (Carcavallo *et al.*, 1998; Lent & Wygodzinsky, 1979; Schofield,

1994). Most species are native to the Americas, although 4 species have

been identified in the Old World. Of

these 7 are related to *T. rubrofasciata* and found in ports around the world in tropical and subtropical regions. *T. rubrofasciata* has a strong ecological association with the rat (*Rattus rattus*), and is the host of the trypanosome *T. conorhini*. This trypanosome has no clinical significance to humans (Gorla *et al.*, 1997).

The major vectors of human Chagas disease are *T. infestans*, *R. prolixus* (Figure 7), *Panstrongylus. megistus*, *Triotoma. brasiliensis* and *T. dimidiata*. *T. infestans* is the single most important vector in the southern cone countries and southern Peru. The only confirmed sylvatic ecotopes so far identified are in Bolivia (Cochabamba region, Bolivia and the Bolivian chaco, Noireau *et al.*, 2000). It is thought *T. infestans* spread with human migration and commerce during the growth of the coffee culture (Dujardin *et al.*, 1987) and has proved supremely suited to domestic and peridomestic environments. The close association of *T. infestans* to domestic environments and

lack of sylvatic ecotopes contributed to the success of the southern cone initiative. *R. prolixus* is the primary vector in northern countries of South and Central America. *P. megistus* is the primary vector along the eastern coast of Brazil, with *T. brasiliensis* common in arid regions of northeastern Brazil. *T. dimidiata* is common in Central America and northwestern regions of South America.

Many species of triatomine have particular ecological niches and host preferences. However some species are far more eclectic in behaviour and host preferences than others. This creates a complex mosaic where information regarding ecological, host, and trypanosome interactions ultimately determines the risk of human infection. (Diotaiuti *et al.*, 1995; WHO, 1991; Schofield *et al.*, 1995,1999; Coura *et al.*, 1999)

#### **1.10.2 Host associations**

Despite such complexity some strong ecological associations can be made (Gaunt & Miles 2000). Of the three most important genera (*Rhodnius*, *Panstrongylus*, *Triatoma*) *Rhodnius* spp are often categorised as palm tree dwellers, with several species feeding on birds and arboreal mammals. They are also vectors of the closely related *Trypanosoma rangeli*. Arboreal mammals such as *D. marsupialis* are the primary hosts of *T. cruzi* in many areas of Latin America with infection rates reported of over 70% in some areas (Travi *et al.*, 1994). The ability of these marsupials to populate peridomestic environments represents an important interface between sylvatic and domestic cycles.

The potential significance of *Didelphis* on disease transmission has increased since the discovery that the vector stages of *T. cruzi* can be sustained in the anal glands. The epidemiological significance of this is unknown (Fernandes *et al.*, 1992; Steindel &

Pinto 1988). Arboreal species of *Panstrongylus* commonly feed from arboreal mammals, however other members of the genus, such as *P. geniculatus* frequent terrestrial burrows and are associated with edentates (such as *D. novemcinctus*). Of the twenty species of *Triatoma* most are terrestrial inhabiting rocky habitats. Exceptions include *Triatoma sordida* which is arboreal preferentially feeding off birds (Lent & Wygodzinsky, 1974; Schofield & Dias, 1999; Crocco & Catala, 1997; Carcavallo *et al.*, 1999; Silveira & Vinhaes, 1999).

Distribution of populations of domiciliated bugs often reflects their natural ecotopes (Gaunt & Miles, 2000). *R. prolixus* can often infest palm roofs (Rabinovich *et al.*, 1995) and *P. megistus* in earth floors, mud walls, or wooden timbers (Nascimento *et al.*, 1997). Populations of the highly domiciliated *T. infestans* have been isolated even in high quality housing with tiled roofs (Schofield 1999).

### **1.10.3 Transmission cycles**

Triatomine species are labelled domestic, peridomestic, or sylvatic, with respect to their ecological niche, and risk to humans. Regions where *T. cruzi* is common in sylvatic hosts with sporadic human infections but no domestic bug colonies are termed the enzootic. It is likely that the oral route of infection is extremely important in sylvatic cycles involving insectivorous hosts such as marsupials, rodents, edentates and primates. Continuous or overlapping transmission cycles occur when the same triatomine species is found in houses and adjacent sylvatic habitats. Definition of whether domestic and sylvatic transmission cycles are overlapping or separate is crucial to the planning of control campaigns. If domestic colonies are replenished from sylvatic foci spraying and surveillance might need to be more frequent. If there are no relevant sylvatic populations re-invasion from this source does not need to be

considered. Examples of such overlapping transmission cycles are found regions of Venezuela where *R. prolixus* infests houses and adjacent palm trees. Localities with a domestic triatomine species confined to houses have discontinuous or separate transmission cycles. At least four of the five highly domiciliated triatomine species have spread beyond their original sylvatic range. The most notable example of is *T. infestans*, which is thought to have originated from rocky areas in Bolivia (Dujardin *et al.*, 1987) and to have spread to all six southern cone countries, and southern Peru. Similarly *R. prolixus* is thought to have spread to Central America (Dujardin *et al.*, 1998), *T. dimidiata* from Central America to parts of northern South America and *P. megistus* from southern Brazil to central and northern Brazil (Gaunt & Miles, 2000). Molecular genetic analyses of triatomine populations and of *T. cruzi* strains can help to define whether there is continuity between domestic and sylvatic transmission cycles.

### **1.11 Genetic diversity of *T. cruzi***

*T. cruzi* has long been presumed to have a genetically heterogeneous population structure. The lack of condensed chromosomes during cell division has prevented characterisation of diversity using electron microscopy, cytological studies; and failed to establish ploidy or the presence of meiotic reproduction (Tait, 1980). The use of pulsed field gel electrophoresis (PFGE) resolved 20-25 chromosomal bands ranging from 0.3 to 1.6 Mb (Henriksson *et al.*, 1993). DNA content has been found to vary even in clones of the same strain (Dvorak *et al.*, 1984).

Evidence for diversity includes the diverse clinical outcomes of infected humans and experimental animals (Andrade, 1999; Tekiel *et al.*, 1997), minicircle fragment pattern or schizodemes (Carneiro *et al.*, 1991; Avila *et al.*, 1990; Morel *et al.*, 1980).

DNA content (Lemesre & Tibayrenc, 1983), oxidative metabolism (Wilkinson *et al.*, 2000), elemental composition of iron, zinc and potassium (Dvorak *et al.*, 1988) drug sensitivity (Neal & van-Bueren, 1988), infectivity to triatomine bugs and mammal hosts (Martins *et al.*, 2000; Phillips & Bertram, 1967), antigenic diversity (Myler, 1993; Snary 1980,1985), and differences in virulence and histotropisms (Basombrio *et al.*, 2000; Cano *et al.*, 2000; Espinoza 1998).

The presumption of heterogeneity was first demonstrated conclusively by isoenzyme electrophoresis (Tibayrenc & Miles, 1983; Miles *et al.*, 1984; Miles, 1985; Cibulskis, 1988). Subsequent profiles using an array of enzyme systems revealed three distinct groups termed zymodemes (Z1, Z2, Z3), with many substrains within each group (Barnabe *et al.*, 2000; Tibayrenc & Breniere, 1988; Tibayrenc *et al.*, 1985).

Recently a plethora of molecular tools have been applied to assess diversity. These include random amplified polymorphic DNA analysis (RAPD, Tibayrenc *et al.*, 1993) which strongly supported strain clusters, polymorphisms in PCR amplified RNA genes and polymorphisms in mini-exon genes (Stothard *et al.*, 1998; Fernandes *et al.*, 1999), microsatellite analysis and functional analysis of gene promoters (Nunes *et al.*, 1997; Oliveira *et al.*, 1998), and sequencing (Machado & Ayala, 2001). The amalgamation of these results indicated the clear presence of two major subdivisions within *T. cruzi*, one equivalent to zymodeme 1 and another encompassing zymodeme 2. A change of nomenclature (Anon, 1999) has since renamed the two intraspecific subdivisions as *T. cruzi* I (TC1) and *T. cruzi* II (TC2) respectively.

The issue of diversity is further complicated by recent analysis based on large datasets from MLEE, RAPD, and characterisation of large and small subunit rRNA and mini-exon genes. These suggest the major lineage, TC2, is further divided into

five smaller phylogenetic subdivisions, designated IIa to IIe (Brisse *et al.*, 2000). This further supports the relevance of genetic units for *T. cruzi* strain classification.

A relatively consistent and coherent picture has emerged. The current view of the subspecific taxonomy of *T. cruzi* is as follows (Brisse *et al.*, 2000):

***T. cruzi* I (TC1)**, equivalent to isoenzyme phenotype Z1

***T. cruzi* II (TC2)**

*T. cruzi* IIa, isoenzyme phenotype Z3

*T. cruzi* IIb, isoenzyme phenotype Z2

*T. cruzi* IIc, isoenzyme phenotype Z3/Z1 ASAT (aspartate aminotransferase)

*T. cruzi* IId, isoenzyme phenotype Bolivian Z2

*T. cruzi* IIe, isoenzyme phenotype Paraguayan Z2

The position of Z3, as *T. cruzi* IIa, within *T. cruzi* II remains controversial, some authors consider Z3 to be more closely related to *T. cruzi* I than *T. cruzi* II (Fernandes *et al.*, 1999) who also separate Z3 into two subgroups, which may correspond to the groups designated *T. cruzi* IIa and IIc. A significant weakness of this view of the subspecific taxonomy of *T. cruzi* is that it is based on isolates collected sporadically across vast geographical distances. The lack of large numbers of isolates from single localities has limited genetic analysis of *T. cruzi* population structures.

There is not complete unity concerning classification, nucleotide sequences from two nuclear genes and a region of the mitochondrial genome do not fully correspond with the two previously defined major lineages instead clustering respectively in three or four distinct clades (Machado & Ayala, 2001), largely because TC2d and 2c partition across TC2b and 2c and are not therefore recognised as distinct clades.

### 1.12 Summary of host/vector *T. cruzi* subdivision associations

Almost all species of the genus *Rhodnius* are primarily associated with palms. The genus *Panstrongylus* is predominantly associated with burrows and tree cavities and the genus *Triatoma* with terrestrial rocky habitats or rodent burrows. Exceptions exist within each genus. Two major sub-divisions have been defined within the species designated TC1 and TC2. TC2 has been further divided into 5 subgroups. Host and habitat associations indicate that TC1 is present in an arboreal, palm tree habitat with the triatomine tribe Rhodniini, in association with the opossum *Didelphis*. The principal geographic range of TC1 is in the vast Amazon basin and countries to the North of the Amazon. TC1 is linked to both sylvatic and domestic cycles. It has been suggested that TC2 is associated with terrestrial habitats in burrows and in rocky locations with the triatomine tribe Triatomini, likely hosts are edentates, rodents or possibly ground dwelling marsupials. TC2 is most commonly isolated from the southern cone. However the associated transmission cycle is so far almost exclusively domestic. Zymodeme 3 has been associated with *P. geniculatus* and burrowing animals such as armadillos. It is rare in humans, and its evolutionary affiliation is unclear (Stothard *et al.*, 1998).

### 1.13 Genotypes and disease prognosis

The correlation between the diverse genetic population structure of *T. cruzi* and clinical outcome of Chagas disease is a key issue often yielding conflicting results. Miles (1985) noted the possibility of differences in clinical outcomes due to Venezuelan and Brazilian strains. Further studies have failed to establish firm evidence of this (Breniere *et al.*, 1989). More recently Andrade & Magalhaes (1997)



noted a correspondence between histopathological profiles in experimentally infected animals and zymodemes, using 138 *T. cruzi* strains. However use of animal models and subsequent extrapolation to human pathology is controversial. Nevertheless it is likely that different *T. cruzi* genotypes are linked to both the pathogenesis of Chagas disease (Vago *et al.*, 2000), and to specific transmission cycles. Geographically Chagas myocardiopathy maintains the same clinical presentation everywhere. However chagasic megaesophagus predominates in Central Brazil. It is relatively rare in other endemic regions and very rare in Central America (Luquetti, *et al.*, 1986; Castro *et al.*, 1994). For the first time TC1 specific and TC2 specific epitopes of a mucin-like antigen (trypanosome small surface antigen, TSSA) have been used to devise a strain-specific serological test by ELISA (Di Noia *et al.*, 2002). All chronic Chagas disease was associated with TC2 seropositivity. However samples were confined to the patients in the southern cone countries and the authors did not evaluate human sera from endemic countries north of the Amazon, where TC1 predominates. Here infection is still associated with myocardiopathy although not with mega syndromes. Furthermore a recent study by Garzon *et al.*, (2002) in Ecuador identified the first observation of chronic chagasic patients infected with Z3, previously only reported rarely in acute infections. This finding shows that previous assumptions concerning clinical and epidemiological traits in relation to genotype may require modification. Future genetic studies on the major subdivisions may yet allow more robust conclusions to be drawn. However the complex zoonosis, heterogenous population structure, socioeconomic factors and differences in host response make it difficult to accurately assess virulence and pathogenesis in humans.

## **1.14 Evolution of trypanosomes**

### **1.14.1 Divergence of the kinetoplastids**

The order Kinetoplastida, which includes both the trypanosomes and bodonids are believed to have diverged early in evolutionary history soon after eukaryotic cells acquired mitochondria through symbiotic incorporation (Gray, 1999).

Origins and the co-evolution of hosts and associated trypanosomes are hotly debated (reviewed by Hoare, 1972). One of two main theories of parasitism proposes that monogenetic trypanosomatids first arose in early insect invertebrates. This was followed by a transition into plants and vertebrates (with the adaptation to haematophagy) evolving into digenetic organisms. The second theory proposes that digenetic organisms first arose in primitive vertebrates, infesting the alimentary tract with transmission by cyst. This was followed by adaptation to blood and intracellular forms eventually leading to transmission by hematophagous invertebrates (Hoare & Wallis, 1966).

### **1.14.2 Choice of phylogenetic marker**

With the lack of palaeontological evidence only recent advances in molecular and phylogenetic analysis have enabled attempts to elucidate evolutionary relationships between extant species (Stevens & Gibson, 1999). These data when combined with other speciation events such as host evolution and biogeographical events aim to provide the answers concerning trypanosomatid evolution. Initial studies focused on human pathogenic trypanosomatids including *Trypanosoma* and *Leishmania* spp. (Maslov *et al.*, 1996; Croan *et al.*, 1997; Lukes *et al.*, 1997). Recently this has expanded to include a large number of species from each genus (Stevens *et al.*, 1999).

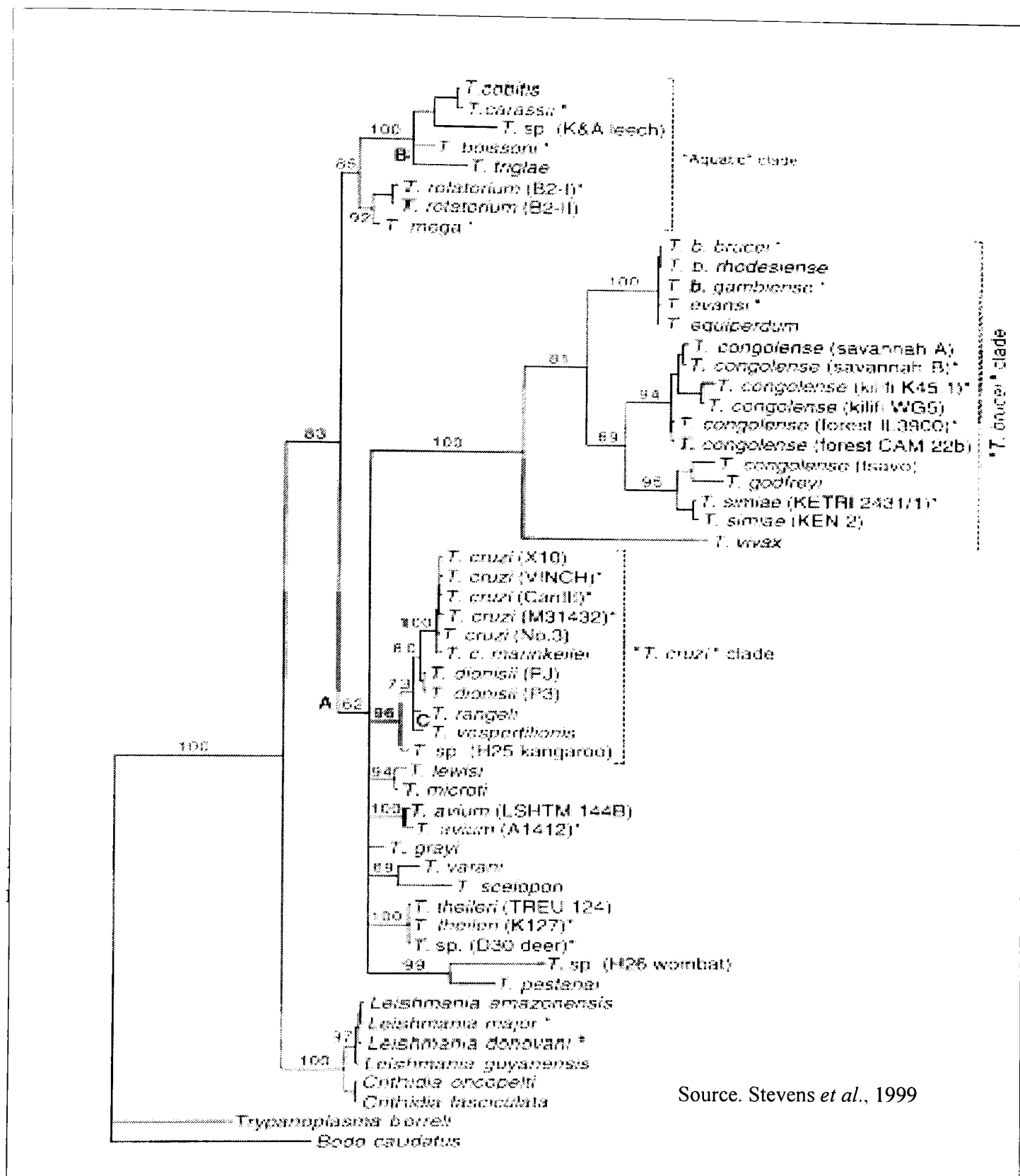


Figure 8 – Trypanosome phylogeny based on the 18S rRNA marker.

The primary marker used in evolutionary studies in the trypanosomatids has been the 18S rRNA marker. This is conserved throughout all eukaryotes, and is present in high copy numbers enabling easy PCR amplification and possesses a range of conserved and variable regions which enable both higher and intra-specific evolutionary studies (Sogin *et al.*, 1986; Stevens & Gibson, 1999). Initial results using

the 18S RNA marker using a limited number of species (*T. brucei*, *T. cruzi* and a fish trypanosome) indicated that the group was paraphyletic (Maslov, 1996). More recent work (Haag *et al.*, 1998; Maslov, 2001) using 24 trypanosome taxa and Stevens *et al.*, (1999) give strong evidence to the genus being monophyletic (Figure 8). This would imply that convergent evolution of trypanosomatids did not occur, but does not discount the development of digenetic life cycles in separate lineages. In contrast a recent study (Hughes & Piontkivska, 2003) using phylogenetic analysis of 18S rRNA sequences from the families Trypanosomatidae and Bodonidae found the section *Salivaria* of the genus *Trypanosoma* fell outside a cluster that included *Stercoraria* of the genus *Trypanosoma*, along with members of the genera *Leishmania*, *Endotrypanum*, *Leptomonas*, *Herpetomonas*, *Phytomonas*, *Crithidia*, and *Blastocrithidia*. This suggested that parasitism of vertebrates has probably arisen independently a number of times within the Trypanosomatidae. The debate of monophyletic or paraphyletic origins Trypanosomatidae remains unresolved.

A variety of other markers have been used including trypanothionine reductase (Alvarez *et al.*, 1996), elongation factor 1 $\alpha$  (Hashimoto *et al.*, 1995), 19S and 12S mitochondrial rRNA (Lake *et al.*, 1998) all indicate that trypanosomes are monophyletic. Other rRNAs (such as the 28S rRNA) are in broad agreement with 18S rRNA. Interestingly analyses of the protein coding gene GAPDH show a monophyletic trend even when 2 to 5 trypanosome species are used (Wiemer *et al.*, 1995; Alvarez *et al.*, 1996) and could be used as a more reliable indicator of resolving time scales of trypanosome evolution in future studies (Stevens & Gibson, 1999).

### 1.14.3 Problems encountered in using molecular markers in evolutionary studies

One of the main problems encountered using the 18S rRNA marker is the high rate of substitution in the *T. brucei* clade as compared to those in other *Trypanosoma* clades. This leads to unequal evolution rates and a distortion of tree topology (Stevens & Gibson, 1999). To some extent this can be corrected for by the appropriate choice of outgroups using various closely related sister genera. Additionally more studies within each genus can help clarify phylogenetic relationships. For instance the association of *T. cruzi* and *T. rangeli* has been confirmed by mini-exon sequences (Stevens *et al.*, 1999). Other phylogenetic considerations include consistency of sequence alignment, a large number of automated computer programmes are available, which vary in their alignment consistency (Morrison & Ellis, 1997; Ellis *et al.*, 1998). Additionally some sites, which are informative between taxa, can introduce “noise” at higher levels as the frequency of non-evolutionary similarity increases (homoplasy) resulting in lower bootstrap values.

### 1.14.4 Dating trees and splits

Three main methods are used in dating trees and splits. The molecular clock approach (Zuckerkandl & Pauling, 1965) assumes sequence changes based on constant mutation rates. There has been much criticism levelled at this method concerning the consistency of sequence changes (Wilson *et al.*, 1987). Using estimates of 0.85% substitutions per 100 million years Escalante & Ayala (1995) estimated the split between the salivarian and other trypanosomes at 300 Mya. The second method accounts for congruence of host and parasite phylogenies. Here parasite trees are calibrated against known time points within host phylogenies obtained from independent records such as fossils. Using this method Haag *et al.*

(1998) dated the split between salivarian and other trypanosomes at 260-500 Mya. A major assumption when using this method is that present host/parasite relationships reflect the past. In reality host switching may occur and interfere with analysis (Mitter *et al.*, 1991). The third method commonly used is in the context of biogeographical events (vicariance biogeography), Nelson & Rosen (1981). Stevens *et al.* (1999) dated the divergence of the salivarian clade and other trypanosomes to the mid Cretaceous period (100 Mya) when Africa became separate from other continents (Smith *et al.*, 1994). At this time mammals were present but not diverse. Other studies have reached similar conclusions based on palaeoecological data (Lambrecht, 1980). Interestingly *T. brucei* associated with mammal hosts and tsetse vectors appear far removed from amphibian and reptile species (*T. mega* and *T. varani* respectively).

#### **1.14.5 *T. cruzi* intraspecific evolution**

Concerning *T. cruzi*, the clade is strongly associated with mammals of South America. Efforts have been made to relate the dispersal and evolution of *T. cruzi* to hosts, vectors and geography (biogeography) (Briones *et al.*, 1999; Kawashita *et al.*, 2001). However the absence of fossil records, controversy over molecular dating methods and incomplete historical vector data make this a formidable task. In contrast the origins and dispersal of South American mammals has been well documented (Simpson, 1980). From 100 to 38 Mya South America was an isolated island continent. Native fauna included marsupials, edentates and ungulates. Rodents and primates colonised from N. America 38 Mya (Oligocene), by island hopping. Approximately 5 Mya (Pliocene) South and North America was connected by the Panama isthmus enabling free migration of fauna. Placentals entered South America.

and a small number of marsupials, edentates and caviomorph rodents (Norwak-Ronald., 1991) re-entered North America.

Kawashita *et al.* (2001) proposed two evolutionary theories (Figure 9) based on biogeographical data and applying 18S rDNA molecular clock dating using variable substitution rates (between 0.85% and 2-4% divergence per 100 My). Using this method *T. cruzi* is categorised into four clades termed riboclades. Riboclade one corresponds to TC1, with riboclades 2, 3 and 4 contained within TC2. The first theory states that riboclade 1 evolved separately over 84 Mya and was introduced with placental mammals after connection of the Americas. This occurred after connection of the Americas in the Pliocene (5Mya ago) or with the arrival of caviomorph rodents and primates during the Oligocene (38 Mya ago). The second theory proposes that all riboclades evolved within S. America during the end of the Cretaceous and Cenozoic. Placental mammals were subsequently infected 37 Mya during the Pleistocene.

In a third theory Briones *et al.* (1999) proposed that TC1 and TC2 lineages diverged between 88 and 37 (Mya) based on rDNA. They hypothesise that lineage TC2 arrived recently with North American placental mammals.

It is possible that the ancestral hosts of these trypanosomes were marsupials or their predecessors. Hemiptera were present 300Mya from fossil records (Briones *et al.*, 1999) and could have been the original insect vector at this time. The only members of the clade found in the Old World are those of bats which are not restricted by geographical barriers. Importantly the inclusion an Australian marsupial



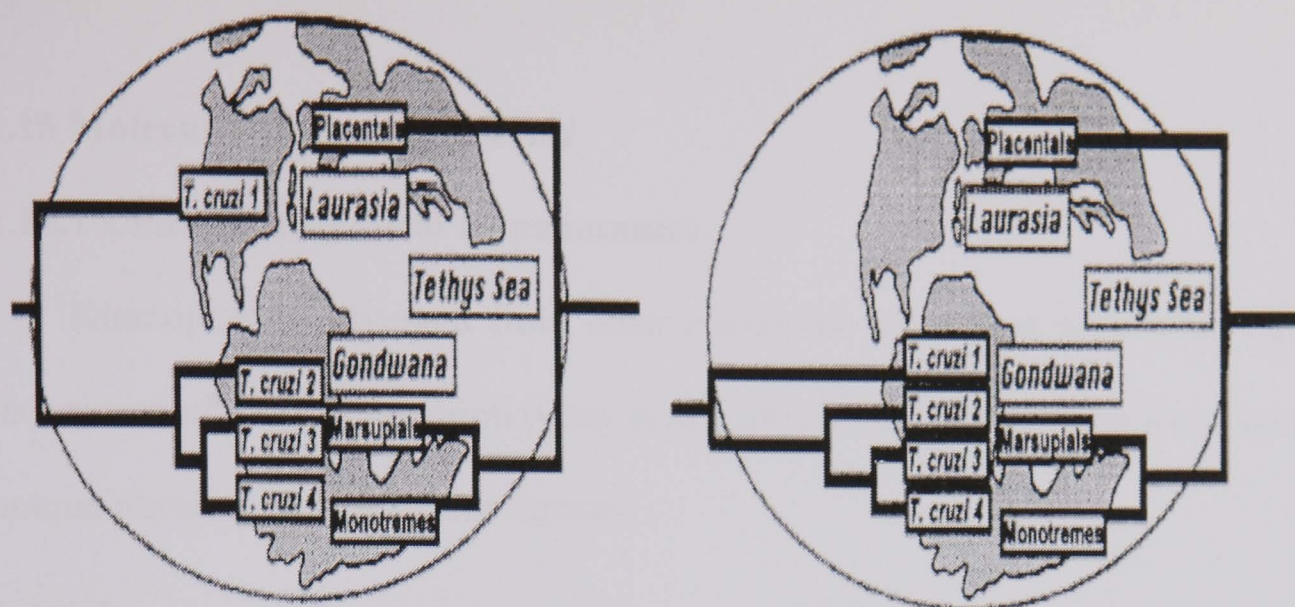


Figure 9 - *T. cruzi* evolution (A) Assumes 0.85% per 100 Myr divergence rate. (B) Assumes 2-4% divergence rate for the 18S rRNA gene. (A), TC1 evolves separately during 84 Mya and after the reconnection of the Americas in the Pleistocene-Pliocene, placental mammals that invaded South America introduced TC1 (Briones *et al.*, 1999). In scenario (B), TC1, TC2 (including associated subgroups) diverged within South America during the end of Cretaceous and the Cenozoic, evolving with insect vectors. Placentals such as rodents and primates became infected after their invasion of South America 37 MYA or during the Pleistocene-Pliocene faunal exchange with North America. Figure taken from Kawashita *et al.*, 2001.

trypanosome within the *T. cruzi* clade reinforces the possibility that it arose in the supercontinent of S. America, Antarctica and Australia which split from Africa approximately 100 Mya (Stevens & Gibson, 1999) during the mid-Cretaceous.

#### 1.14.6 Trypanosome evolution, the human context.

From a human perspective it appears that clades containing *T. brucei* and *T. cruzi* have very different evolutionary histories with very different time scales. In Africa *T. brucei* has an ancient co-evolution with primates and tsetse flies spanning at least 15 million years (Stevens & Gibson, 1999) and with *Homo* spp. dating at least 3 mybp (Stevens *et al.*, 1998). In contrast human exposure to *T. cruzi* is relatively recent and did not occur before the arrival of humans estimated at 30-40,000 years ago. According to Rothhammer (1985) there is no evidence of infection before permanent human settlements prior to 3000 years ago. It is likely that human infections were additions to the host range including other primates (d'Alessandro & Saravia, 1999).



## 1.15 Molecular biology of *T. cruzi*

### 1.15.1 Unusual features of trypanosomes

Kinetoplastids diverged from other eukaryotic organisms soon after symbiotic incorporation of mitochondrion (Gray *et al.*, 2001). This may account for a number of unique physical and molecular aspects.

### 1.15.2 Kinetoplast

Unlike other eukaryotes, trypanosomes, contain a single mitochondrion and visible mitochondrial DNA housed in a structure known as the 'kinetoplast' (Simpson, 1973). The mitochondrial genome 'kinetoplast DNA' consists of a network of concatenated minicircles (5000, each 0.46-2.5kb, Figure 10) exhibiting significant heterogeneity and approximately 50 genetically homogenous maxicircles (20-38kb) (Avila, 1995). The reason for the evolution of two kinds of DNA circles is unknown. Transcripts are modified by a novel process termed 'RNA editing'. Maxicircles are the equivalent to the mitochondrial genome of other eukaryotes containing a number of genes coding for particular constituents of the mitochondrial respiratory chain. Unlike other eukaryotes many of these genes of the maxicircles of the kDNA are incomplete and require editing of their transcribed RNA in order to give rise to a functional protein product (Sloof & Benne, 1997). Editing is performed utilizing a large number of guide RNAs, these are encoded by the many slightly different minicircles (Macina *et al.*, 1985). In contrast to maxicircles they are heterogenous in nucleotide sequence and evolve rapidly (Vickerman, 1994)



Figure 10 – Electronmicrograph of catenated minicircles in the kinetoplast. Source, [www.rnaucle.edu/trypanosome](http://www.rnaucle.edu/trypanosome)

### 1.15.3 Unusual RNA editing in trypanosomes

RNA editing in *T. cruzi* and other trypanosomes involves the insertion and deletion of uridine residues (U's) in maxicircle transcripts (Benne, 1990). Most gRNAs are encoded in the thousands of minicircle molecules which are concatenated together into a single giant network of DNA.

Sequence heterogeneity of the kinetoplast minicircle DNA (Avila & Simpson, 1995) raised several issues. Until relatively recently it was unclear as to how minicircle DNA in *T. cruzi* can have any gRNA-coding capacity at all. There do not appear to be any variable region sequences conserved between different strains. The question was resolved by comparing sequence data from maxicircle gRNAs obtained from two clonal strains. Libraries of minicircle variable regions were constructed by PCR amplification. A gene was identified for each gRNA in the same polarity within specific minicircle variable regions. Total gRNA from one strain failed to cross hybridise with the other. This was due to the number of polymorphisms existing in each of the homologous DNA sequences. Despite these polymorphisms the integrity of the mRNA/gRNA was maintained in most instances due to G-U wobble base pairing. Avila & Simpson (1995) concluded that the variable sequences comprising homologous gRNAs between the strains were derived from an ancestral sequence. Within a strain, multiple redundant gRNAs were identified which encode identical editing information but possess different sequences.

Practical applications of these novel mechanisms have been exploited. The presence of these parasite-specific but highly polymorphic DNA fragments from kinetoplast minicircle molecules can be detected by PCR amplification of DNA from patients chronically infected with *T. cruzi* (Sturm *et al.*, 1989; Avila *et al.*, 1993;

Avila & Simpson, 1995). Schizodeme analysis of minicircle regions after PCR amplification has also been demonstrated to provide sensitive resolution, detection and classification of *T. cruzi* stocks (Avila *et al.*, 1993).

It remains unclear why kinetoplasts require such a complex RNA editing system. One hypothesis is that RNA editing corrects defects in the maxicircle gene and represents an alternative method of handling genetic information. Stuart & Feagin (1992) suggested that editing is regulated to produce different forms of mRNAs in response to different selection influences. In this way RNA editing could be a way of modifying or changing gene expression.

#### **1.15.4 Transcription**

Another unique aspect, with respect to other eukaryotes, is that trypanosomatid DNA is transcribed continuously as polycistronic messenger units (Elias *et al.*, 2001, Xu *et al.*, 2001), through a novel trans-splicing reaction all mature mRNAs receive a 36-nucleotide-long leader sequence: the so-called "spliced leader" or "mini-exon". This spliced leader is joined to the 5' end of most gene transcripts (McCarthy *et al.*, 1989) Spliced leader genes have been shown to exhibit remarkable intrastrain homogeneity, but significant interstrain heterogeneity (McCarthy *et al.*, 1989).

### **1.15 5 Mitochondrial activity**

Mitochondrial activity is required for oxidation of amino acids during passage through the triatomine vector and utilising the functional respiratory chain and citric acid cycle. Carbohydrates are limited in the triatomine gut although high concentrations of amino acids exist as a product of digestion of the bloodmeal.

Trypanosome mitochondria, have the usual set of cytochromes including cytochrome oxidase, cytochromes b, c, c1 and o (Schwarcz, 1985). For cytochrome c, in contrast to the cytochrome c of other organisms, the haem is attached by one rather than by two cysteines to the protein (de Meirelles & Souza, 1982). Cytochrome oxidase catalyses the interaction of reducing equivalents produced during respiratory oxidation reactions with atmospheric oxygen. Interestingly, in the case of *T. brucei* electron transfer is performed not by cytochromes but by a cyanide-insensitive glycerol 3-phosphate oxidase.

### **1.15.6 The glycosome**

The mitochondrion works in conjunction with another organelle unique to the kinetoplastids called the glycosome (Vickerman, 1994). This structure houses the enzymes of the glycolytic pathway. In contrast with these enzymes eukaryotes these are absent from the cytosol. It is believed that this compartmentalisation enables different forms of the life cycle to operate more efficiently than usual in eukaryotic cells. This is particularly important for some species (for example *T. brucei*) which is dependent on glycolysis for energy production in the bloodstream form. Other reported functions of the glycosome include carbon dioxide fixation, oxidation of fatty acids, and pyrimidine salvage (Vickerman, 1994)

### **1.15.7 Surface membranes**

Additional unique features of trypanosomes include the complex nature of the surface membrane. Most glycoproteins or glycolipids are inserted into the plasma membrane by a glycosyl phosphatidyl inositol (GPI) anchor. Functions of these vary widely between species, some performing specific functions and others contributing to a general protective glycocalyx (Almeida *et al.*, 1994). Concerning *T. cruzi* surface structure is complex with stage specific glycoconjugates. Relatively recently GPI anchored glycoproteins have been discovered with trans-sialidase activity on the surface of trypomastigotes. The function of this enzyme is to transfer acid residues from the host to the trypomastigote surface. This is implicated in cell entry into non-phagocytic host cells (Villalta *et al.*, 2001).

### **1.15.8 Nuclear apparatus of *T. cruzi***

Unusually chromosomes do not condense at mitosis and can only be resolved by pulse field electrophoresis (section 1.9). Homologues of pairs may be of different sizes, and heterologues the same size. Trypanosome genomes evolve rapidly due to transposable elements and high rates of homologous recombination. Gene duplication may also play an as yet undefined role (Lynch & Conery, 2000) This limited or optional role of sexual reproduction has allowed homologous chromosomes to evolve separately.

### 1.16 Current issues in the molecular biology of *T. cruzi*.

Many recent advances have been made in the basic molecular biology and the more unusual aspects of *T. cruzi*. Diverse issues encompass variations in gene expression in different developmental stages (Ruef *et al.*, 1994), glycoconjugate structure and function (Colli & Alves, 1999), the organisation and expression of multigene families encoding for virulence (Carmo *et al.*, 1999), as well as global questions concerning the epidemiology and evolution and pathogenicity of *T. cruzi* relating to diversity (Bastrenta *et al.*, 1999; Robello, 2000; Oliveira *et al.*, 1998 ).

A major technical breakthrough in molecular biology has been the development of genetic transformation systems using plasmid and cosmid shuttle vectors. This has enabled large sections of *T. cruzi* DNA (up to 45 kb) to be propagated in bacteria and reincorporated into the parasite, either episomally or by homologous recombination (Kelly *et al.*, 1994). This has revolutionised the analysis of gene structure and function as genes can be manipulated, deleted or overexpressed.

Much interest has recently focused upon the mechanisms of metacyclogenesis using differential gene expression (Krieger *et al.*, 1999). The primary objective is to isolate and characterise the genes expressed at terminal stages of differentiation during metacyclogenesis. A technique (representation of differential gene expression, RDE) allows direct comparison of expressed genes in closely related populations; PCR amplified specific gene sequences are isolated by subtractive hybridisation using oligonucleotides representing mini-exon and polyA sequences as primers. By the use of RDE, genes have been identified at different stages of metacyclogenesis. Some genes are transitionally expressed, others continuously expressed specifically in metacyclic forms (Avila *et al.*, 2001).

Mechanisms of cell entry by *T. cruzi* have also been extensively studied in recent years (Colli & Alves, 1999). Of particular relevance are glycoconjugates contained in the plasma membrane of *T. cruzi*. Several categories exist. One such group consists of mucin like glycoproteins which cover the entire surface of the epimastigotes and trypomastigotes. It has been estimated that  $3 \times 10^6$  molecules are present on the surface of epimastigotes and  $1.5 \times 10^6$  on the surface of trypomastigotes. They are the major substrates of trans-sialidase, a secreted enzyme linked to host invasion (Colli & Alves, 1999). These molecules have influence on adhesion, penetration of the host cell and escape from the phagolysosome into the cytoplasm (Burleigh & Andrews, 1998). Isolated glycoproteins have been shown to contain O-linked oligosaccharides, terminally substituted with numerous sialic acid units. It is the unusual characteristics of the lipid moiety of GPI anchors, the presence of acidic residues, which affect the immunological properties of the molecule. Mucin characteristics have been demonstrated to differ in epimastigote, metacyclic and cultured trypomastigote forms in terms of molecular mass, susceptibility to proteinases and glycosidic structure (Yoshida *et al.*, 1993). Of particular interest, trypomastigote mucins have been identified (Almeida *et al.*, 1999) which induce synthesis of proinflammatory cytokines IL-12, TNF- $\alpha$ , and nitric oxide production by macrophages. This has physiological relevance, *in vivo*, as these factors may be shed into the surrounding medium by infective forms. In addition, the terminal  $\alpha$ -galactopyranosyl units in oligosaccharides have been found to be targeted by antibodies isolated from patients with Chagas disease. From this it is possible to monitor the decrease in titres by chemiluminescent ELISA in patients given chemotherapy (Almeida *et al.*, 1994).

Other issues include genomic organisation and expression of surface antigen multigene families (Reuf *et al.*, 1994). Genes encoding gp90 and gp82 are of



particular interest. Both have been implicated in host cell penetration and have surface antigens anchored via the GPI anchor. Gp82 has been demonstrated to induce a  $\text{Ca}^{2+}$  signal essential for internalisation of the parasite (Ruiz *et al.*, 1998). Immunisation of mice with the glycoproteins has been shown to protect from a virulent strain of *T. cruzi* (Planelles *et al.*, 2001; Yoshida *et al.*, 1993). Homologues of these genes have been identified in epimastigote forms and also in *T. rangeli* (Briones *et al.*, 1995) suggesting a common ancestral gene. The aforementioned lines of research are by no means comprehensive but indicates the diverse issues that are currently researched.

### **1.17 Genetic exchange in trypanosomes**

Tibayrenc *et al.*, (1990) first proposed the theory of clonality in parasitic protozoa, and initiated the debate on the relative importance of sexual and clonal reproduction (Andrade, 1999, Laurent *et al.*, 1997; Tibayrenc & Breniere, 1988). To date the only trpanosomatid which has been demonstrated experimentally to undergo genetic recombination is *T. brucei*, the causative agent of African sleeping sickness (Gibson, 1996; Gibson & Stevens, 1999; Sternberg & Tait, 1990). The first success was by Jenni *et al.* (1986) using two cloned trypanosome populations (247-L and 386-AA). Tsetse flies (*Glossinidae spp.*) were fed through a membrane with a mixture of the putative parental types and subsequently allowed to feed on mice. Clones representing the parental types and subsequent hybrids were isolated. The mechanism for exchange probably involves meiosis. This deduction was based on reassortment of genetic markers (isoenzyme, RFLP) with, high frequencies of chromosomal recombination relative to parental clones (performed by pulse field gel electrophoresis), and hybrids containing a DNA content of  $2n$  and  $3n$  (Gibson, 2001). The frequency of genetic recombination in *T. brucei* remains speculative both in terms of experimental data and population genetics analysis (Sternberg *et al.*, 1989).

In addition the mechanisms of genetic recombination are poorly understood, and although consistent with meiosis (Gibson & Bailey, 1994) haploid stages have not been observed and resolution of other putative, intermediate, stages remains controversial.

Experimental crosses of other *Trypanosoma* species have been unsuccessful although naturally occurring putative homozygous and heterozygous isolates have been characterised by isoenzyme analysis and DNA markers. Natural hybrid combinations of molecular characters have been described in New and Old world *Leishmania* spp. (Evans *et al.*, 1987; Kelly *et al.*, 1991; Darce *et al.*, 1991; Belli *et al.*, 1994) and also putative parents and production of *T. cruzi* hybrids (Stothard *et al.*, 1999). Evidence of historical genetic exchange in *T. cruzi* was produced by Machado and Ayala (2001) who used phylogenetic analysis of two nuclear genes and one mitochondrial gene, showing that genetic exchange had occurred between lineages and that two strains (including CL Brener) are in fact hybrids. Further details specific to genetic exchange in *T. cruzi* can be found in section chapter 4.0.

### **1.17.1 Approaches used to detect genetic exchange**

Two main approaches are used in the assessment of genetic exchange, experimental studies, and population genetics with evolutionary studies (Gibson & Stevens, 1999). With regard to the latter the aim is to measure gene flow within and between populations. This is achieved by testing for the presence of subdivisions within populations where gene flow is minimal or absent. This follows the collection of isolates, followed by molecular characterisation and the use of appropriate population or phylogenetic methods. The development of such techniques has only been feasible recently since the development of molecular methods including

isoenzyme, RFLP analysis, PCR based applications and more recently sequencing in conjunction with rapid advances in computer technology.

Methods for the analysis of genetic recombination in protozoa fall into three main categories. Segregation tests (Tibayrenc *et al.*, 1985, 1986; Gibson & Miles, 1986) are based on Hardy Weinberg equilibrium, examining random assortment of different alleles at a given locus. This requires identifiable alleles and a ploidy above one. Examination of chromosomes in *T. brucei* indicate that larger chromosomes are diploid, but analysis of smaller chromosomes is inconclusive (Tait 1980; Tait *et al.*, 1989; Borst *et al.*, 1982). With respect to *T. cruzi* the issue of ploidy is incompletely resolved. Many studies, based primarily on heterozygous isoenzyme profiles, molecular karyotypes and DNA content reveal heterozygous profiles which have been interpreted as diploid hybrids (Tibayrenc & Breniere, 1988; Bogliolo, 1996). However direct studies reveal a complex situation, in relation to the huge variability which exists in genetic content and arrangement. Dvorak (1984) observed a 30 - 70% difference in total DNA content in clones, suggesting aneuploidy. Similarly Gibson & Miles (1986) observed differences in chromosome size and organisation concluding that *T. cruzi* was, at the minimum, a diploid organism. More recently microsatellite studies (Oliveira *et al.*, 1998) are in agreement with diploidy in *T. cruzi*.

Recombination and linkage methods are multilocus analyses and can be utilised at any ploidy level. Loci studied must be independent from one another (Tibayrenc 1995, 1996) and demonstrate a deviance from random assortment.

Phylogenetic methods have classically been used to examine higher echelons of evolutionary divergence. Recently these techniques have been applied to address evolutionary questions where species boundaries are confused (Tibayrenc, 1996). Cibulskis (1988) examined a range of trypanosomatids for genetic exchange using

Wagner networks. Results indicate that, in agreement with experimental observations, *T. brucei* exhibited a degree of exchange, but not *T. cruzi*. More recently Machado & Ayala (2001) provided the first proof of historical genetic exchange in *T. cruzi*. The presence or absence of genetic exchange could have significant implications for disease control. In a clonal model individuals consist of independently evolving lineages. Such clones may be quite different in their biological and therefore pathogenic properties. In panmictic populations a common gene pool exists, an individual is genetically ephemeral (Tibayrenc *et al.*, 1991) In this instance it is the population that needs characterising. The contentious issue of genetic exchange specific to *T. cruzi* is discussed in chapter 3.

### **1.18 Summary**

Many molecular studies indicate a complex, largely clonal population structure which exhibits remarkable inter-strain and intra-strain heterogeneity. Molecular markers have grouped *T. cruzi* into two main groups or lineages (TC1 and TC2), one circulating primarily in the southern cone principally in domestic cycles (TC2) and another primarily throughout the vast Amazon basin and countries North of the Amazon in both sylvatic and domestic cycles (TC1). Recently one of these lineages (TC2) has been further subdivided into five smaller phylogenetic subdivisions based on MLEE/RAPD and rRNA/mini-exon markers. However recent gene sequence data from mitochondrial and nuclear gene sequences cluster *T. cruzi* in three or four distinct clades respectively, not fully corresponding to the two predefined major lineages. Recent evidence suggests the presence of genetic exchange. The mechanism and implications of genetic exchange in *T. cruzi* are unresolved. To some extent

diversity can be correlated to strain virulence, host association, susceptibility to drugs, and more controversially pathology.

### **1.19 Aims of the project**

1. To optimise a solid medium technique for isolating biological clones of *T. cruzi* from vectors and mammals and to examine the extent of multiclonality in populations from individual triatomine vectors and mammal hosts, in Paraguay and elsewhere.
2. To contribute to a re-examination of the degree of genetic diversity of the major lineage *T. cruzi* II (TC2) in Paraguay, and the association with vector and mammal species.
3. To contribute to experimental studies of genetic recombination of *T. cruzi*.

## **2.0 MATERIALS & METHODS**

### **2.1 Trypanosome stocks**

#### **2.1.1 Stocks used in analysis of genetic variability**

*T. cruzi* stocks included in the present study were isolated from various sources in Brazil and Paraguay. *T. cruzi* was isolated from sylvatic bugs in Brazil. In Paraguay isolates were acquired from domestic and sylvatic cycles, from triatomine vectors and mammals from different locations. Strains and sources used in specific analysis are defined in each of the results sections. Reference strains were obtained from the cryobank of the London School of Hygiene and Tropical Medicine.

#### **2.1.2 Stocks for experimental genetic exchange analysis**

Initial studies identified putative parental and hybrid strains from the single locality of Serra de Carajas in the Brazilian Amazon basin (Carrasco *et al.*, 1996). During previous work using these strains, the two putative parental lines were genetically transformed to be resistant to different drugs. Parental types PI was transfected with pGAP-Hyg conferring resistance to Hygromycin B (150ug/ml) and PII transfected with pTEX conferring resistance to G418 (Neomycin, 120ug/ml). PI-pGAPHyg was sensitive to G418, PII-pTEX was sensitive to Hygromycin B.

Natural isolates were also used in this study to detect historical genetic recombination. Stocks and methods are described fully in chapter 4.0.

## 2.2 Collection of triatomine bugs and the capture of sylvatic mammals

An array of methods were used to acquire triatomine bugs. Sylvatic triatomine bugs were collected from their natural ecotopes by ‘microhabitat dissection’ (Miles *et al.*, 1981), the use of live baited Noireau traps (Abad Franch *et al.*, 2000), and by the co-operation of local inhabitants. Briefly, Noireau traps (Figure 11) were constructed from a screw-capped plastic container with a wire mesh incorporated into the lid.



Figure 11 – Noireau trap with two captured adult *R. prolixus*

Over this is placed a fine mesh of synthetic material (for example fine nylon curtain mesh) preventing ants from entering the trap. A single mouse was placed inside the container with cotton wool as bedding, a piece of fruit for moisture and a small amount of mouse food. When sealed the

exterior of the trap was covered with double sided sticky tape. Triatomine bugs, attracted to heat and carbon dioxide emitted by the mouse became trapped on the tape. Traps were laid in the evening, collected in the morning and placed in a variety of locations including palm trees (Figure 12A, typically four per tree), bromeliads, rock crevasses, tree holes and under fallen trees.

Bugs were also obtained from domestic and peridomestic habitats by manual searches. In some locations the supervised participation of members of the local communities was utilised for the manual capture of domestic and sylvatic bugs by microhabitat dissection. Palm tree dissection (Figure 12B) was performed by felling





Figure 12A, Placment of Noireau traps in a palm tree (*Attelea butyracea* ). 12B, Dissection of a felled palm tree. 12C, Palm tree containing numerous hanging birds nests (unidentified).

the tree followed by systematic dissection and searching palms from the base of the tree to the crown and leaves. A 10m palm took approximately 90 mins to dissect. Care was taken to examine any rodent or birds nests found in trees (Figure 12C).

Mammals were acquired by the use of collapsible ‘live-traps’ baited with a mixture of peanut butter, ripe banana and oats mixed into a paste. Traps were placed along animal runs indicated by trodden pathways, near burrow entrances or dense vegetation. Traps were set at sunset and examined in the morning shortly after sunrise. Some animals, particularly armadillos were unsuitable for capture by animal



such instances local hunters were hired to collect live specimens, which were processed and released unharmed.

### **2.3 Isolation of trypanosomes from triatomine bugs**

This protocol is described fully in section 3.4.3.

### **2.4 Isolation of *T. cruzi* from mammal hosts**

Trypanosomes were isolated from mammals using one of three methods.

#### *1. Xenodiagnosis followed by bug dissection and in vitro culture*

Typically 6 bugs were used to xenodiagnose each animal. Bugs were allowed to feed for 20 min. Colony reared *T. infestans* or *R. prolixus* were used for xenodiagnosis. Bugs were dissected three weeks following xenodiagnosis. *In vitro* culture was performed by faecal inoculation (following dissection) into 3ml of diphasic blood agar medium (1.4% (w/v) blood agar, trypticase 0.5% (w/v), NaCl 0.6% (w/v), defibrinated rabbit blood 10% (v/v)), prepared in standard sterile test tubes. The gel was allowed to set for 10 min, then overlaid with either 5 drops of 0.9% sterile saline containing 5-fluorocytosine (150ug/ml) and gentamycin (150ug/ml) or RPMI 1640 medium (GIBCO BRL, Paisley, Scotland) supplemented with 0.5% (w/v) trypticase (BBL), 0.5% (w/v) HEPES, 0.03M haemin, 10% (v/v) foetal calf serum (FCS, heat-inactivated), 2mM sodium glutamate, 2mM sodium pyruvate, and containing gentamycin (150ug/ml) and 5-fluorocytosine (150 ug/ml).

#### *2. Cardiac puncture*

Followed by direct inoculation onto diphasic blood agar medium or direct plating (3.4.1).

### 3. Centrifugation of cardiac blood (triple centrifugation technique)

The triple-centrifuge procedure was used to attempt to detect the presence of trypanosomes in the peripheral blood when parasitaemia was light. Centrifugation was followed by inoculation of sediment into diphasic blood agar medium or direct plating was performed. The protocol is described in section 3.4.4.

## 2.5 Solid media plating technique for isolation of clonal colonies of *T. cruzi*

Isolation of clonal colonies of *T. cruzi* was performed to identify the optimum growth conditions. The experimental procedure is described fully in chapter 3. Briefly *T. cruzi* isolates from whole blood or dissected bug gut contents were added to low melting point agarose (LMP, Sigma) maintained at 37°C and containing antibiotics and antifungals. This solution was poured onto pre-prepared nutrient underlays contained in a petri dish (Figure 13). Different nutrient underlays and LMP overlays were compared to determine the combination that allowed for the fastest growth, most viable colonies and highest plating efficiencies using reference strains. Plates were sealed with parafilm (American National) and incubated at 28°C for up to 3 months.

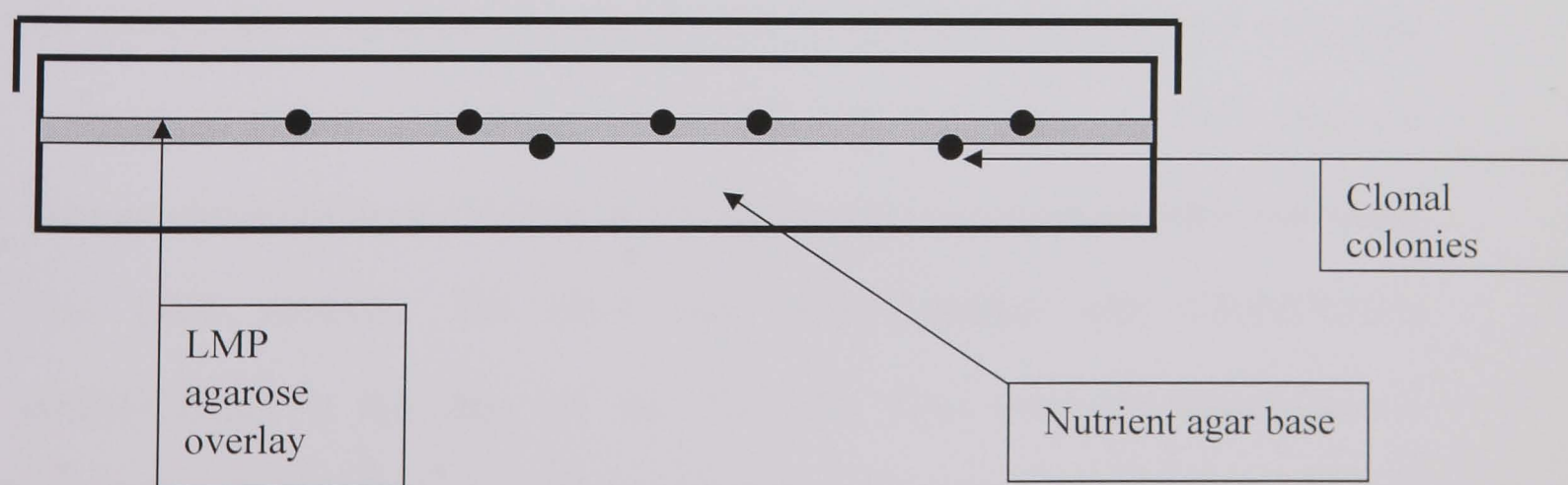


Figure – 13 depiction of clonal colonies growing in the LMP agarose layer of the solid media plate.

## 2.6 Harvesting of clonal colonies from agarose plates

Mature colonies were visible to the naked eye and were harvested directly using a sterile pipette tip. Smaller colonies could be visualised under an inverted microscope *in situ* to monitor colony growth. Using this method, single cells could be viewed. The isolated colonies were placed in a 26 well tissue culture plate (Nunclon) containing 1ml of supplemented RPMI 1640 medium (2.4) and incubated at 28°C until the culture reached logarithmic growth phase. Further expansion was achieved by the addition of 1ml of culture into 10ml of fresh supplemented RPMI medium contained in a 25ml tissue culture flask.

## 2.7 Preparation of total DNA

Total DNA was prepared from 10ml of *T. cruzi* culture in exponential growth phase ( $3-5 \times 10^7$  cells/ml). Cells were harvested by centrifugation at 3000g at 4°C for 10 min, and the supernatant discarded. Cells were washed once in chilled PBS (pH 7.2) and resuspended in 1ml of lysis buffer (50mM NaCl, 50mM EDTA, 1% SDS, 50 mM Tris, pH 8.0). Proteinase K was added to a final concentration of 100µg/ml, and the mixture left to incubate overnight at 37°C. Suspensions were extracted with equal volumes of phenol, phenol/chloroform (1:1) and chloroform. At each stage the mixture was centrifuged (20,000g at 4°C for 10 min) and the upper phase containing total DNA removed. The DNA was further purified with GENECLAN (GENECLAN II Kit, Bio 101 Inc., La Jolla, CA.) using the manufacturer's guidelines. High purity total DNA required for sensitive applications such as sequencing or RAPD (random amplified polymorphic DNA) was isolated using

DNeasy kits (Quaigen) or DNase (Bioline) kits following the protocols provided by the manufacturer.

### **2.7.1 Measurement of DNA integrity and concentration**

Following DNA extraction, samples were tested for purity and integrity. To determine DNA integrity 2µl of sample was run on a 0.7% agarose gel, stained with ethidium bromide and visualised with UV light. Single discrete bands were an indicator of good quality DNA. DNA quantitation was performed by electrophoresis of samples in parallel with DNA standard markers (Bioline, UK). The purity of stock DNA was assessed by measuring the absorbance at 260 and 280nm (1  $A_{260}$ =50 mg/ml dsDNA). A ratio of absorbance between 1.8 and 2.2 indicated an acceptable level of purity.

## **2.8 Harvesting of epimastigotes for isoenzyme analysis**

Log phase parasites (8ml) were harvested by centrifugation at 3000g for 10min at 4°C, washed once in ice cold PBS (0.14M NaCl, 2.7 mM KCl, 10mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2). The pellets were resuspended in an equal volume of enzyme stabiliser (2mM EDTA; 2mM dithiothreitol). Suspensions were lysed by freeze thawing 3 times in liquid nitrogen. Lysates were centrifuged at 13,000rpm at 4°C for 30 min. 15µl aliquots were added dropwise into liquid nitrogen forming discrete beads which were collected and stored in liquid nitrogen until required.

### **2.8.1 Isoenzyme electrophoresis conditions**

Thin layer starch gel electrophoresis (Miles *et al.* 1980a, 1980b, 1981) and cellulose acetate electrophoresis (Lanham *et al.*, 1981), was performed. Enzymes tested were

aspartate aminotransferase (E.C.2.6.1.1. ASAT); glucose phosphate dehydrogenase (E.C.1.1.1.49, G6PD); glucose phosphate isomerase (E.C.5.3.1.9. GPI); phosphoglucomutase (E.C.2.7.5.1, PGM) and 6-phosphogluconate dehydrogenase (E.C.1.1.1.44, 6PGD).

## **2.9 Random amplified polymorphic DNA (RAPD)**

Reaction conditions were similar to those described by Carrasco *et al.*, (1996). Each 20µl reaction mixture contained 10mM Tris-HCl (pH8.8), 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.001% gelatin, 0.2mM of each of 2'-deoxynucleoside 5'-triphosphate, dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology, Uppsala, Sweden), 20 pg of primer, 1.0 unit of *Taq* DNA polymerase (Stratagene, La Jolla, CA), and 0.5-20ng of parasite genomic DNA. The reaction mix was overlayed with 40µl of mineral oil (Sigma Chemical Co., St. Louis, Mo) and amplified in a Hybaid thermal reactor (Hybaid Ltd., Middlesex, United Kingdom). Primers A1, A4, A6, L4, L5 and H1 (Table 2.1) were previously shown to distinguish between the three principal zymodemes (Carrasco *et al.* 1996). In Addition 5 primers (Table 2.1) were used which had previously been reported to resolve six *T. cruzi* phylogenetic lineages (Brisse *et al.*, 2000).

The reaction conditions were as follows. Two cycles at 95°C for 5min, 30°C for 2 min and 72°C for 1 min, 32 cycles at 95°C for 1 min, 40°C for 30 sec, and 72°C for 2 min; with a final cycle with extension at 72°C for 5 min. Reaction products were electrophoresised on a 2% agarose gel (Sigma Chemical Co., St Louis, Mo) in 1 X TAE buffer (0.04 M Tris-acetate, 0.001M EDTA), stained with ethidium bromide, and photographed on an ultraviolet transilluminator.



**Table 2.1** Nucleotide sequences of primers used in RAPD genotyping.

Primer Name	Sequence
L4*	5'-GTGGATGCGA
L5*	5'-AAGAGCCCGT
H1*	5'-CGCGCCCGCT
A1*	5'-TCACGATGCA
A2*	5'-GAAACGGGTG
A3*	5'-AGTCAGCCAC
A4*	5'-AATCGGGCTG
A6*	5'-GTGATCGCAG
A15	5'-TTCCGAACCC
F5	5'-CCGAATTCCC
R16	5'-CTCTGCGCGT
U7	5'-CCTGCTCATC
U14	5'-TGGGTCCCTC

\* Primers used in characterisation of experimentally derived TC1 hybrids.

## **2.10 PCR amplification of the non-transcribed spacer of the mini-exon gene**

Characterization of the mini-exon was performed (Souto *et al.*, 1996) using a pool of primers TC (5'-CCC CCC TCC CAG GCC ACA CTG), TC1 (5'-GTG TCC GCCACC TCC TTC GGG CC), and TC2 (5'-CCT GCA GGC ACA CGT GTG TGT G) and the same reaction concentrations as the 24S $\alpha$  rRNA characterizations. Cycle amplification was performed in a Hybaid thermal cycler with 27 cycles (30 sec 94°C, 30 sec at 55°C, 30 sec at 72°C) followed by a final elongation of 5 min at 72°C. PCR. Products (of size 300 or 350 bp) were separated by electrophoresis in 1.5% agarose gels for 90 min at 90V in 0.5X TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

## **2.11 PCR amplification of the size-variable domain of the 18S rRNA sequence**

Characterization of the 18S rRNA fragment was performed using primers V1 (5'-CAA GCGGCT GGG TGG TTA TTC CA) and V2 (5'-TTG AGG GAA GGC ATG ACA CAT GT), as described by Brisse *et al.* (2000). These primers target a size-variable domain corresponding to positions 994 (3' flank base) to 1164 (5' flank base) of the *T. cruzi* Peru strain sequence. Reaction conditions were the same as for the 24S $\alpha$  rRNA characterizations. Cycle amplification was performed in a Hybaid thermal cycler with 30 cycles (1 min at 94°C, 1 min at 50°C, 1 min at 72°C) followed by a final elongation step of 5 min at 72°C. PCR products were separated by electrophoresis at 80V during 2 h in 3% agarose gels with 0.5 X TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

## **2.12 PCR amplification of the D7 divergent domain of the 24S $\alpha$ rRNA**

Characterization of the 24S $\alpha$  rRNA was performed following the method described by Souto *et al.* (1996) using primers D71 (5'-AAG GTG CGT CGA CAG TGT GG) and D72 (5'-TTT TCA GAA TGGCCG AAC AGT). Amplification reactions were performed in a final volume of 25 $\mu$ l containing 10mM Tris-HCl pH 9.0, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01 % gelatin, 0.1 % Triton X-100, 2 mM of each dNTP, 20 pM of each primer, 0.5 units of *Taq* DNA polymerase (Stratagene, La Jolla, CA), and 20ng of purified trypanosome DNA. Cycle amplification was performed in a Hybaid thermal reactor (Hybaid Ltd., Middlesex, United Kingdom) during 30 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) followed by a final elongation step of 5 min at 72°C. Amplified products were separated by electrophoresis at 80V over 2h in 3% agarose gels (Sigma Chemical Co., St Louis, Mo) with 0.5X TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

## **2.13 Size estimation of the PCR amplification products**

The sizes of the 24S $\alpha$  rRNA and 18S rRNA PCR amplification products were estimated after electrophoresis on 3% agarose gels using a DNA molecular weight marker containing reference fragments sizes of 75 bp, 100 bp, 134 bp, 154 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp and 1,000 bp. The size markers were contained in the 1 kb DNA ladder (Bioline) and the 100 bp DNA ladder (Bioline). In addition profiles were assessed using gel imaging and calibration software (Labworks, Version 4.0.0.8, UVP Inc., Upland, CA)

## 2.14 Southern blotting and hybridisation of filters

Digested DNA was size fractionated on 0.8% agarose gels by using 1xTAE buffer and stained with 0.5 mg/ml ethidium bromide. In preparation for membrane transfer agarose gels were bathed in a depurination solution for 15 mins (0.25M HCl) followed by denaturation in 0.5M NaOH - 1.5M NaCl for 30min. The gel was then placed in a neutralising buffer (1.5M NaCl - 1.0M Tris-HCl pH 7.2) for one hour. DNA was transferred to nylon membranes (Hybond-N; Amersham) using 20xSSC (3M NaCl, 0.3M sodium citrate) buffer as described by Southern (1975). On completion the DNA was covalently bound to the membrane by exposure to UV (Stratalinker, stratagene). Membrane filters were blocked in prehybridisation buffer (Southern, 1975) at 42°C for 3-24hrs and hybridised with hybridisation buffer (Southern, 1975) containing the corresponding oligo-labelled DNA probe, at 42°C overnight. DNA probes were radiolabelled with  $^{32}\text{P}$ -dCTP (Amersham), using a random primer DNA labelling kit (*rediprime*, Amersham) according to the manufacturer's instructions. Each membrane was washed at increasing stringency until overall counts had fallen significantly indicating the removal of non specifically binding radioactive probe. Typically each filter was washed for 15min in 2xSSC at room temperature and in 0.2xSSC at 60°C for 30–60 min. Membranes were then exposed to X-ray film (Kodak XAR-5 film) at -80°C.

## **2.15 Sequencing reactions**

Several primers were used in the amplification of isoenzyme gene loci and their sequencing (Table 2.2). Those for glucose phosphate isomerase (GPI) were designed by S Obado (personal communication). Phosphoglucumutase (PGM) and Pteridene reductase primers were designed by MW Gaunt (personal communication). Mitochondria primer sequences were obtained from Machado & Ayala (2001), and amplified a region encompassing cytochrome oxidase and NADH dehydrogenase.

Table 2.2 Table to show the names, function and sequence of primers used.

Name	Primer Function	Sequence
SO1	Forward GPI	GGC ATG TGA AGC TTT GAG GCC TTT TTC AG
SO2	Reverse GPI	TGT AAG CGC CCA GTG AGA GCG TTC GTT GAA TAG C
GPI.seq.for	Forward GPI	CGC ACA CTG GCC CTA TTA TT
GPI.seq.rev	Reverse GPI	TTC CAT TGC TTT CCA TGT CA
GPI.564	Internal GPI	TGT GAA GCT TTG AAG CCT TT
GPI.467	Internal GPI	GGT CAG GAG AGG TGA ATG GA
PGM.for	Forward PGM	GGG CGG AAC TAC TAC TGT CG
PGM.rev	Reverse PGM	GGA GGG AGT AAA AGA AAG GAA AA
IG1	Forward PR	GGT SGA CAT GCT CGG TGT GC
IG2	Rev PR	AAS CTT CAG TCC GCA CTC GTG
ND1.3A	Fwd mitochond	GCT ACT ART TCA CTT TCA CAT TC
COII.2A	Rev mitochond	GCA TAA ATC CAT GTA AGA CMC CAC
COII.A400	Internal mitochond	CTC CTA TTA CAA CCA ATA AAC ATC
Mito.425	Internal mitochond	ATG CCG TCT GTA ATA GGT GTC A
Mito.850	Internal mitochond	ATC CAC AAA TTT TGA TGA TAT A
Mito.950	Internal mitochond	CAA AAT TTA AAC AAC CGA AAT ATA

## 2.16 PCR amplification reactions

Amplification reactions were followed according to those specified in respective publications in the case of published primers. Reaction components and conditions were optimised in the case of designed primers. Final reaction volumes were 50µl and



amplification reactions used the following conditions: denaturation for 5min at 94°C. then 30 cycles of 94°C (1min), 50–62°C (1 min; depending on the primer T<sub>m</sub>) and 72°C (1min per 1000 bp), followed by 10 min at 72°C.

## **2.17 PCR product purification**

PCR products were purified using the QIAGEN<sup>TM</sup> PCR purification kit following the manufacturer guidelines. Briefly, five volumes of high chaotrophic salt buffer (PB) were added to one volume of PCR reaction product followed by centrifugation. This resulted in the binding of product to a silica membrane housed in a 'QIA quick column'. A washing process using an ethanol based buffer was performed followed by further microcentrifugation to remove any trace ethanol. DNA bound to the silica membrane was then eluted using 30-50µl of elution buffer. In those instances that DNA concentration was low a MiniElute (Qiagen) spin column was used which provided a more concentrated DNA volume (10µl)

## **2.18 Gel purification of PCR products**

PCR products were obtained and purified from agarose gels, prior to sequencing using, using the QIAGEN<sup>TM</sup> gel purification kit. This was necessary to remove products present as a result of non-specific primer binding. A 1% agarose gel was prepared with 1X TAE buffer. Samples were loaded and electrophoresed for approximately 1 hour at 90V, stained with ethidium bromide and visualised on an ultraviolet transilluminator. The appropriate band was identified by comparison with a molecular weight ladder, excised and placed in a pre-labelled 1.5ml microfuge tube. Three volumes of buffer QG (containing high salt concentrations and a pH indicator) were added and the contents incubated for 10 min at 50°C in order solubilise the

agarose. The solution was then applied to a QIA quick minicolumn. Agarose was removed using a buffer (QG) and DNA extracted, washed and eluted according to the PCR purification protocol (see above).

## 2.19 Cloning

Cloning of specific sequences was achieved utilising the protocol set out for pGEM®Teasy plasmid (Figure 14), JM109 (high efficiency) *Escherichia coli*

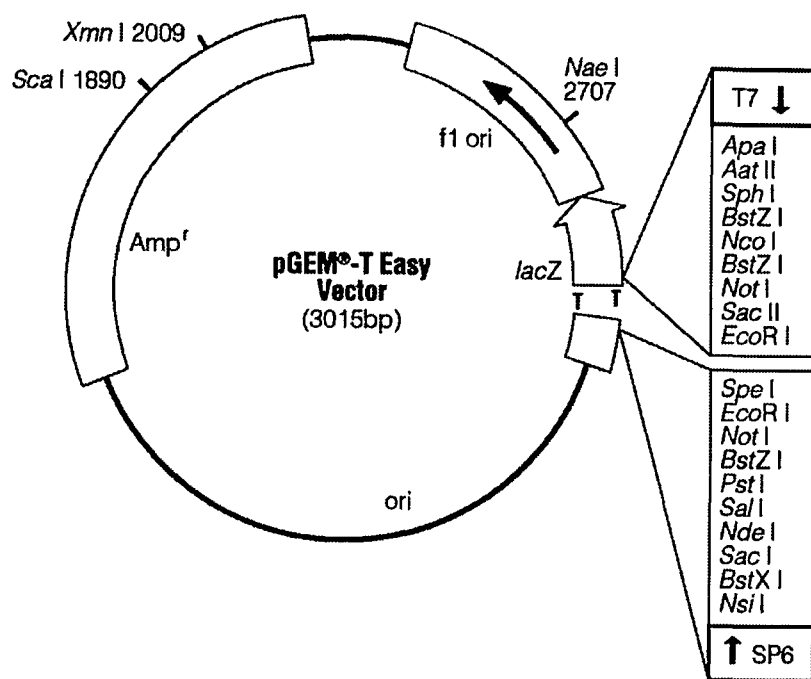


Figure 14 - pGEM® Easy Vector circle map

competent cells, ampicillin and  $\beta$ -galactosidase (X-gal) screening (Promega). The ligation mixture was prepared on ice and included 5 $\mu$ l of 2x T4 DNA ligase rapid ligation buffer, 1 $\mu$ l of pGEM®Teasy vector, 1 $\mu$ l T4 DNA ligase and 3 $\mu$ l of PCR product optimised for a product

vector ratio of 1:1. The ligation reaction was mixed by gentle pipetting and left a 4°C overnight. The ligation makes use of the unusual property of *Taq* polymerase to add adenosine (A) residue to the end of a PCR product. The plasmid can be cut to expose a tyrosine (T) residue enabling the PCR product to ligate. X-gal screening was a visual indicator of a successful ligation. On transformation into competent cells, if ligation is unsuccessful and the PCR product is not inserted into the plasmid, the *lac Z* gene is able to function producing a blue colony. Hence if ligation is successful the point of insertion interrupts the *lac Z* gene (Figure 14) and the colonies appear white.

To transform the plasmid into competent cells, 5µl of the ligation reaction was added to 50µl of JM109 high efficiency *E. coli* cells and gently mixed and left on ice for 20 min. This allowed the plasmid to associate with the outer membrane of the cells. The cells were then heat shocked for 45 seconds at 42°C and returned to ice for two minutes. The heat shock process causes flipping of the cell membrane and subsequent plasmid internalisation. Cells were added to a tube containing 950µl of SOC medium and placed on a shaker at 150rpm, 37°C for 1.5 hours. Each transformation was then plated onto LB plates containing ampicillin, IPTG and X-gal. As the plasmid confers resistance to ampicillin but the JM109 cells do not, non transformed cells are selected against. Plates were incubated overnight at 37°C. To certify the presence of a cloned gene, five white colonies from each transformant were selected at random and a screening PCR was performed. Primers SP6 and T7 (Supplied with the Promega pGEM®Teasy kit) were used and possessed specificity for sites located in the linker regions of the plasmid (Figure 14). Products were visualised under UV on a 1% agarose gel with ethidium bromide. Usually the PCR products were of adequate quality for sequencing. However in some instances PCR fragments were not of adequate quality, due primarily to non-specific binding. In this case further purification was needed (2.21).

## **2.20 Plasmid purification**

QIAGEN™ QIAprep spin miniprep kit (Qiagen) protocol was used to harvest high endpoint DNA concentrations for sequencing. Briefly transformed colonies were removed from the plate with a sterile pipette tip and grown in 5ml of LB broth incubated at 37°C overnight in a mechanical shaker. Following this 3ml of the broth was pelleted in a 1.5ml microfuge tube (3000rpm for 15 min) after which the cells

were resuspended in a buffer containing RNAase A. Cells were then lysed with an alkaline buffer and the solution gently homogenised to prevent shearing. Following a 5 min incubation period a high salt buffer was added to neutralise the reaction and enabled the binding of DNA to the silica spin column. Cell debris was removed by microcentrifugation and the resultant supernatant was applied to a QIAprep spin column. Once applied the DNA was washed with ethanol based solution and eluted.

## 2.21 Sequencing

Sequencing was performed using an ABI 377 sequencer, MWGAG Biotech Primus 96+ thermal cycler and samples prepared using an ABI Prism<sup>®</sup> Bigdye<sup>™</sup> Terminator cycle sequencing ready reaction kit. (Applied Biosystems). The protocol

Table 2.3. Cycle sequence reaction volumes.

Reagent	Quantity
Terminator Ready Reaction Mix	4µl
PCR template	5-29 ng
Primer	3.2 pmol
Deionised water	<i>q.s</i>
Total Volume	10µl

for the sequencing of plasmids and PCR products was followed. Big dye reaction mixtures were prepared, containing dideoxynucleoside triphosphate dye terminators (ddNTPs), dNTPs, AmpliTaq<sup>®</sup> DNA polymerase, MgCl<sub>2</sub> and buffer. 3.2pmol of primer and an 5-29ng of template DNA (Table 2.3). Final reaction volumes were 10µl. Reaction conditions were as follows. Rapid thermal ramp to 96°C, 96°C for 10 s, rapid thermal ramp to 50°C, 50°C for 5s. rapid thermal ramp to 60°C, 60°C for 240s.

The cycle was repeated 25 times. Rapid thermal ramp equated to 1 °C/sec. Samples were cleaned by isopropanol precipitation, following the ABI Prism<sup>®</sup> protocol, before being pelleted and dried at 90°C for 1 min in order to remove excess isopropanol which would otherwise inhibit the reaction. DNA was resuspended in loading buffer and electrophoresed using an ABI sequencer which determined the position of the incorporated dye terminators

## **2.22 DNA sequence assembly and comparison**

Sequencing was tracked by using ABI Prism 377 software. Sequences were then imported to an auto-assembler package to be edited and aligned using ABI Prism Sequence Navigator<sup>™</sup> Version 1.0.1 (Perkin –Elmer) using Clustal V (Higgins, 1994) and Bioedit (Hall, 1999). Regions of poor sequence resolution were deleted following which, forward reverse and internal (if appropriate) sequences aligned and a consensus sequence obtained. This approach in conjunction with manual examination of sequence data was used to identify regions of interest.

## **2.23 Isoenzyme and RAPD data**

Isoenzyme bands and RAPD profiles were scored for by presence (1) or absence (2). RAPD profiles were scored using Labworks image analysis software (Version 4. UVP inc., Upland, CA) verified by manual checks. Bands that were faint or those with poor amplification profiles were excluded from the analysis. Pooled data was analysed using PHYLIP (Felsenstein, 1993) and JMP (Version 4.0.2, SAS Institute inc.).

### 3.0 OPTIMISATION OF THE ISOLATION OF CLONAL COLONIES OF *T. cruzi* ON SOLID MEDIA FROM AXENIC MIXTURES, MAMMALS, AND TRIATOMINE BUGS

Previous methods of isolating *T. cruzi* clones from bugs and mammals are time consuming and often ineffective. Historically this is based on the isolation of single organisms by direct microscopy, dispensing individual organisms via capillary tube onto coverslips (Miles, 1974), or by limiting dilution. Sources of isolates have included whole blood, axenic cultures or triatomine bug faeces. Single organisms are then placed in culture medium. Examination of cultures typically occurs 14 days after incubation at 28°C. The relative inefficiency of this method was demonstrated by Miles (1974) who obtained 2 clones from 28 inoculated cultures (using *T. cruzi* strain 7).

Cloning on solid medium has been described for several parasitic protists including *Entamoeba histolytica*, *Giardia lamblia* (Gillin & Diamond, 1978) and the insect flagellates *Crithidia hamosa* and *Strigomonas megaseliae* (Keppel & Janvoy, 1972). Additionally *Leishmania* (Gambarelli & Dumon, 1988) and *T. brucei* (Carruthers & Cross, 1992) have been successfully grown on plates.

With regard to *T. cruzi*, clonal isolation has been cited in a limited number of publications as having much potential for aiding research into many aspects of the parasite. Goldenberg & Chiari (1980) first demonstrated the growth and isolation of single colonies of *T. cruzi* on solid medium. They recognised the potential for the isolation of mutants and for screening antitrypanosomal activity using different chemotherapeutic agents. From the human perspective the importance of resolving clones was demonstrated by Morel *et al.* (1980) who detected a naturally occurring mixed human infection by schizodeme analysis of 11 clones. Mondragon *et al.* (1998) attempted to optimise growth on agar plates using three strains and adapted the



method to enable the screening of genetically transfected clones (Kelly *et al.*, 1992) resistant to the antibiotic G418 thus initiating its application in transfection studies and expression of recombinant genes. Additionally Santos *et al.* (2000) demonstrated that stably transfected epimastigotes could be selected and cloned on solid medium.

The methods previously used in clonal isolation are broadly similar, focusing primarily on obtaining clones from epimastigote cultures. Goldenberg & Chiari (1980) used 0.75 – 1.5% agar fortified with LIT growth medium (liver infusion-tryptose). Epimastigote flagellates were spread over the surface of the dried plates (in 0.1ml LIT), sealed in a plastic bag and incubated. Various other nutrients have been tried including, BLAB medium (0.75% agar supplemented with LIT medium 48.4%, BHI 48.4% and 2.45% defibrinated human blood) and RPMI 1640 (GibcoBRL) based medium. Again inoculum was applied to the surface of the dried plates.

### **3.1 Results of previous solid media plating experiments**

The plating efficiencies of previously published methods vary widely depending on the methods used. These range from 5% (Goldenberg & Chiari, 1980), using CL-Brener and Y stock, to 87% (Gomes *et al.*, 1991) using an uncharacterised strain. Colonies typically become visible after 20 -35 days depending on the strains used and typically reached a diameter of 1mm. The primary morphological forms reported were epimastigotes. Plating efficiencies were also reported to be affected by the number of cells used in the inoculum (Mondragon *et al.*, 1998). Different agars produced different rates of growth which has been partially attributed to sulphate content. Higher sulphate contents (> 0.4-0.7%) produced weaker gels. Gels that were too refined produced media that were too firm and inhibited growth (Santos *et al.*, 2000). Solidification time and temperature has been reported to influence the make up of the

polymer formation (Kin & Yaphe, 1972). Growth phase appeared important with mid to late logarithmic growth producing the most colonies (Mondragon *et al.*, 1998). This finding was in contrast to that of Goldenberg & Chairi, (1980) who found no correlation with growth phase. Once colonies became visible they could be picked off and expanded in fresh culture medium.

### 3.2 Experimental rationale

The rationale for developing this technique further was based primarily on the need to isolate effectively clonal colonies of *T. cruzi* from different sources, with the potential of obtaining many varied strains, known to exist sympatrically in some geographical regions such as Paraguay (Chapman *et al.*, 1984). Different strains of *T. cruzi* have been proven to vary dramatically in their respective doubling times and subsequent growth rates. Examples of doubling times *in vitro* are 6 days (CAN III), 22 hrs (CL-Brener), 16 hrs (X10/6) (Mondragon *et al.*, 1998). Prior to this PhD programme only one previous attempt has been made to grow colonies of strains representative of each of the two main lineages and the sublineage (Z3). Mondragon *et al.* (1998) used such a selection of strains but were unsuccessful in propagating CANIII. They concluded that modifications to the method would be necessary to propagate slow growing strains. A primary obstacle to cloning *T. cruzi* results from this relatively slow rate of growth. Gels eventually desiccate and inhibit growth. In contrast *T. brucei* and *Leishmania* form discrete colonies in 5-7 days (Mondragon *et al.*, 1998). Furthermore it has been noted that in our geographical areas of study in Paraguay up to 50% of isolates from domestic triatomine bugs did not grow in LIT medium (Rojas de Arias, personal communication) and were lost.

Desiccation stops growth but can be partially solved by the use of a humidified incubator (Mondragon *et al.*, 1998), *T. cruzi* growth is also inhibited by basic pH. In rapidly dividing cultures organic acids produced by glycolysis prevent this inhibition. With un-sealed plates gaseous exchange prevents acidification. The use of a 4% CO<sub>2</sub> incubator may facilitate maintenance of an appropriate pH range.

### 3.3 Objectives

Primary objectives were to develop a simple and effective method for isolating clones from a wide range of sources (triatomine bugs and mammals) and to prove that the method has the ability to detect multiclonal infections from these sources. This would provide an essential basis for further genetic characterisation of field isolates of *T. cruzi*. At the time of the initial experiment 5 sylvatic *Rhodnius robustus* were available to resolve multiclonality in insect vectors. Importantly the technique must be suitable for widespread use in Latin America where only minimal facilities exist in many laboratories. No previous publications have directly compared nutrient types and incubation conditions or demonstrated the use of this technique to resolve mixed infections directly from triatomine bug faeces or mammals. Thus experimental objectives were:

1. To develop a sensitive plating technique enabling the growth and isolation of individual clones from representative strains.
2. To demonstrate the isolation of clones from infected triatomine bug faeces and from mammals.
3. To show the technique could resolve multiple infections from triatomine bugs and mammals
4. To achieve high plating efficiencies.

5. To demonstrate growth without the need for a CO<sub>2</sub> incubator, not available in many Latin American laboratories.
6. To produce plates that were resistant to desiccation for slow growing strains
7. To demonstrate high transfer efficiencies for clonal expansion.

### **3.4 METHODS**

#### **3.4.1 Optimisation of plating techniques for isolation of clonal colonies of *T. cruzi***

Isolation of clonal colonies of *T. cruzi* involves the preparation of nutrient fortified agar plates. The principal goal of effective clonal isolation is to prepare plates that stimulate growth but inhibit cell motility. Avoidance of contamination from symbiotic bacteria, yeast from bug intestinal contents and prevention of desiccation are of utmost importance.

An experiment was initially designed to compare different growth media with the goal of optimising growth conditions for clonal isolation. Secondly, growth and plating efficiencies were compared on plates maintained in a CO<sub>2</sub> enriched atmosphere (4%) to growth in a non CO<sub>2</sub> enriched atmosphere. Thirdly plating efficiencies of different strains representing the two major lineages and the as yet equivocally placed lineage (Z3) were compared. The method was initially tested for efficiency using laboratory grown strains, then tested for feasibility using isolates from dissected silvatic triatomine bugs, and finally from the blood of silvatic mammals and infected laboratory mice. Note that in future text RPMI refers to supplemented RPMI (section 2.4). Similarly DIFCO refers to diphasic blood agar medium described in the following section (3.4.2.1)

### **3.4.2 Preparation of solid media plates**

#### **3.4.2.1 Preparation of the underlay**

Plates containing growth media were prepared in two stages. Initially an agar base was prepared and fortified with nutrients. Secondly an overlay consisting of low melting point agarose (LMP, Sigma), containing saline or further nutrients and the inocula (diluted axenic cultures, intestinal bug contents, or mammal blood) was added.

Growth of colonies was optimised by a comparison of the following nutrient bases (underlays).

1. Diphasic blood agar medium (DIFCO) consisting of blood-agar 1.4% (w/v), trypticase 0.5% (w/v), NaCl 0.6% (w/v), fortified with 10% (v/v) whole defibrinated rabbit blood
2. Diphasic blood agar medium fortified with 10% lysed, whole defibrinated rabbit blood. Whole blood was lysed by freeze thawing in liquid nitrogen three times.
3. Diphasic blood agar medium fortified with 10% defibrinated rabbit blood which has been lysed and centrifuged (3000 rpm, 10min) to remove cell debris.
4. Diphasic blood agar medium fortified with 10% (v/v) foetal calf serum.
5. 2.2 ml of 3% Bacto agar fortified with 10.8 ml of supplemented RPMI (Sigma).

12ml of each of the above solutions was poured into separate petri dishes and allowed to set at room temperature for 10 min. Plates were sealed with parafilm (American National) and stored at 4°C in readiness for the overlay. Prior to the addition of the overlay the plates were warmed to ambient temperature.

### 3.4.2.2 Preparation of the overlay

Two types of overlay were used in plate preparations. The first overlay consisted of 0.9% sterile saline containing antibiotics (100µg/ml 5-fluorocytosine and 100 µg/ml gentamycin). The second consisted of supplemented RPMI-1640 (methods 2.4) with antibiotics (100µg/ml 5-fluorocytosine and 100µg/ml gentamycin). This final overlay consisted either of 2.4 ml of saline or supplemented RPMI, 0.6ml of 3% LMP and inocula (maximum volume 150µl).

For comparisons in plate optimisation experiments, an inoculum of 1000 trypanosomes from liquid culture was used. The appropriate number of cells (and thereby culture volume) was calculated by use of a haemocytometer (Scientific Laboratory Supplies). Separate plates were inoculated with reference strains, X10/1 (TC1), Esm cl3 (TC2) and CANIII (Z3) which have been previously reported to grow at different rates *in vitro* (Finley & Dvorak, 1993). Cultures in mid log growth phase were used.

The final overlay solution including inocula was maintained at 37°C and poured evenly over the pre-prepared base, allowed to dry at room temperature for 10 min, sealed with parafilm and placed in a humidified incubator at 28°C. Table 3.1 summarises the medium combinations and incubation conditions.

A total of 180 plates were prepared. Each plate type was prepared in triplicate for each nutrient combination (Table 3.1). This was performed for each of the test strain inocula (TC1, TC2 and Z3). Additionally each plate combination and strain was grown in sealed, airtight petri dishes preventing gaseous exchange (sealed plates), and in plates sealed with Micropore tape (Unichem) enabling gaseous exchange and maintained in a humidified incubator with 4% CO<sub>2</sub>. Additionally three plates were prepared for each plate combination with no inocula (18 controls).



Table 3.1. Solid media nutrient combinations.

Plate type	Nutrient base	LMP overlay
1	RPMI	Saline
2	RPMI	RPMI
3	DIFCO + 10% (v/v) rabbit blood	Saline
4	DIFCO + 10% (v/v) foetal calf serum	Saline
5	DIFCO + 10% (v/v) lysed whole blood	Saline
6	DIFCO + 10% (v/v) lysed and centrifuged blood	Saline
7	DIFCO + 10% (v/v) rabbit blood	RPMI
8	DIFCO + 10% (v/v) foetal calf serum	RPMI
9	DIFCO + 10% (v/v) lysed whole blood	RPMI
10	DIFCO + 10% lysed and centrifuged blood	RPMI

Plate combinations were maintained in a humidified incubator with a 4% enriched CO<sub>2</sub> atmosphere at 28°C. Each plate type was performed in triplicate. In addition identical nutrient combinations were prepared and incubated in sealed, airtight, plates. Plate combinations and conditions were repeated for each of the reference strains X10/1 (TC1), Esm cl2 (TC2), and CAN III (Z3).

### 3.4.3 Preparation of *T. cruzi* from sylvatic and domestic triatomine bugs for plating

The following solutions were prepared and equipment assembled.

1. White's solution  
HgCl<sub>2</sub> 0.025 g  
NaCl 0.65 g  
Conc HCl (sp. gr. 1.18) 0.125 ml  
Ethanol (abs) 25 ml  
H<sub>2</sub>O 75 ml
2. Saline (0.9%) antibiotic solution for dissection containing gentamycin (300 µg/ml) and 5-fluorocytosine/ml (300 µg/ml), filter sterilise.
3. RPMI growth medium containing, gentamycin (100 µg/ml) and 5-fluorocytosine (100 µg/ml), shake vigorously to dissolve, filter sterilise.
4. Equipment  
  
Perspex dissection screen  
Sterile microscope slides  
Coverslips  
Fine forceps - 2 prs  
Broad forceps - 1 pr  
Fine spatula  
Microspatula(s)  
70 % ethanol  
70 % ethanol/iodine solution  
Chloros container

Triatomine bugs were immersed for 10 minutes in White's solution and rinsed in 0.9% sterile saline solution (2). Bugs were then dried with a sterile mediwipe. All procedures were performed under aseptic conditions. Bugs were dissected, behind a perspex screen. To perform the dissection the bug was held upside down using a pair of forceps. Using a second pair of forceps the terminal abdominal segment, and gut contents, were drawn out onto a sterile microscope slide on which was placed a drop of saline solution (solution 2). Gut contents were homogenised with a blunt microspatula. Using a sterile pastette the intestinal contents were transferred to a sterile 3ml Bijou containing growth medium (solution 3). The volume of medium in

the centrifuge tube should not exceed 1ml. The solution was then examined for the presence of trypanosomes and cells counted using a haemocytometer. The inoculum was then ready for plating.

#### **3.4.4 Preparation of trypanosome isolates from mammals for plating**

Various methods were used in an attempt to isolate trypanosomes from peripheral blood.

##### *A. Triple-centrifuge procedure*

1. Centrifuge heparinised blood (10 IU/ml heparin) for 10 min at 300 x g.
2. Remove supernatant fluid and transfer to another centrifuge tube.
3. Centrifuge for 10 min at 500 x g.
4. Transfer of supernatant to another centrifuge tube.
5. Centrifuge for 10 min at 900 x g.
6. Decant the supernatant, and examine sediment as a wet preparation.

##### *B. Whole blood plating.*

This involved the incorporation of whole blood in to the LMP layer. The volumes added ranged from 10-150 $\mu$ l. Larger volumes limited the visibility of embedded individual trypanosomes and colonies due to the number of erythrocytes.

##### *C. Plasma plating.*

Whole blood left to settle for 1hr (in vials containing EDTA as anticoagulant, Monovette, Sarstedt, Germany) or centrifuged at low speed (300 x g, 10 mins), allowing blood cells to settle. Vials were incubated at 37°C for 45mins allowing highly motile trypomastigotes to become distributed in the plasma. Cell counts were performed and aliquots were used to inoculate plates. Inoculation volumes ranged from 10-150  $\mu$ l.

### 3.5 Plating results

#### 3.5.1 Optimisation of growth conditions on agarose plates for representative reference strains

Table 3.2 and Figures 16-19 summarise the results (Appendix 1 shows additional raw data from different plate combinations and incubation conditions). Standard deviation values for growth on duplicate plates are shown graphically in figures 16, 17, 18, and 19. Overlapping comparison circles with an angle of intersection less than  $90^\circ$  denote significance at a 95% confidence interval. No colony growth occurred on the controls and these are excluded from summary tables and comparative analysis.



Figure - 15 *T. cruzi* colony on a solid nutrient plate. Note the epimastigotes on the edge of the colony (thin arrow) and a high density of amastigotes and epimastigotes further from the edge (bold arrow) each cell is approximately 20 microns.

Table 3.2 A summary of growth comparisons for three reference strains with different substrates and incubation conditions.

<table><tr><td>X10/1 (TC1)</td><td>ESM cl2 (TC2)</td><td>CANIII (Z3)</td></tr></table>									X10/1 (TC1)	ESM cl2 (TC2)	CANIII (Z3)
X10/1 (TC1)	ESM cl2 (TC2)	CANIII (Z3)									
PLATE LABEL	PLATE TYPE	INCUBATION CONDITIONS	FIRST VISIBLE COLONY * (DAYS)	PLATING EFFIC (%)	FIRST VISIBLE COLONY (DAYS)	PLATING EFFIC (%)	FIRST VISIBLE COLONY (DAYS)	PLATING EFFIC (%)			
1	RPMI underlay + saline overlay	CO <sub>2</sub>	11	54	22	22	32	5			
		Sealed	18	34	27	23	32	2			
2	RPMI underlay + RPMI overlay	CO <sub>2</sub>	11	58	19	38	29	22			
		Sealed	19	36	24	42	26	8			
3	DIFCO (10% whole blood) + saline overlay	CO <sub>2</sub>	9	64	19	36	31	21			
		Sealed	18	55	21	27	36	14			
4	DIFCO (10% FCS) + saline overlay	CO <sub>2</sub>	18	4	none	0	none	0			
		Sealed	26	12	none	0	none	0			
5	DIFCO (10% lysed whole blood) + saline overlay	CO <sub>2</sub>	9	52	24	6	none	0			
		Sealed	20	40	27	9	none	0			
6	DIFCO (10% lysed and centrifuged Blood) + saline overlay	CO <sub>2</sub>	10	52	19	9	none	0			
		Sealed	19	43	none	0	none	0			
7	DIFCO (10% whole blood) + RPMI overlay	CO <sub>2</sub>	9	75	19	32	27	26			
		Sealed	18	61	22	21	26	17			
8	DIFCO (10% FCS + RPMI overlay)	CO <sub>2</sub>	13	12	27	0	none	0			
		Sealed	24	2	none	0	none	0			
9	DIFCO (10% lysed blood) + RPMI overlay	CO <sub>2</sub>	10	62	20	4	29	0			
		Sealed	19	14	32	7	none	0			
10	DIFCO (10% lysed and centrifuged blood) + RPMI overlay	CO <sub>2</sub>	9	63	24	11	33	1.6			
		Sealed	19	44	35	12	none	0			

\* First visible colonies (>0.2mm) are defined as those visible to the naked eye, when held under an overhead light source against a dark background.

Figure -15 shows a typical colony consisting of epimastigotes towards the edge of the colony and amastigotes towards the centre. Trypomastigotes were also occasionally visible in Esm cl2 plates in mature colonies.

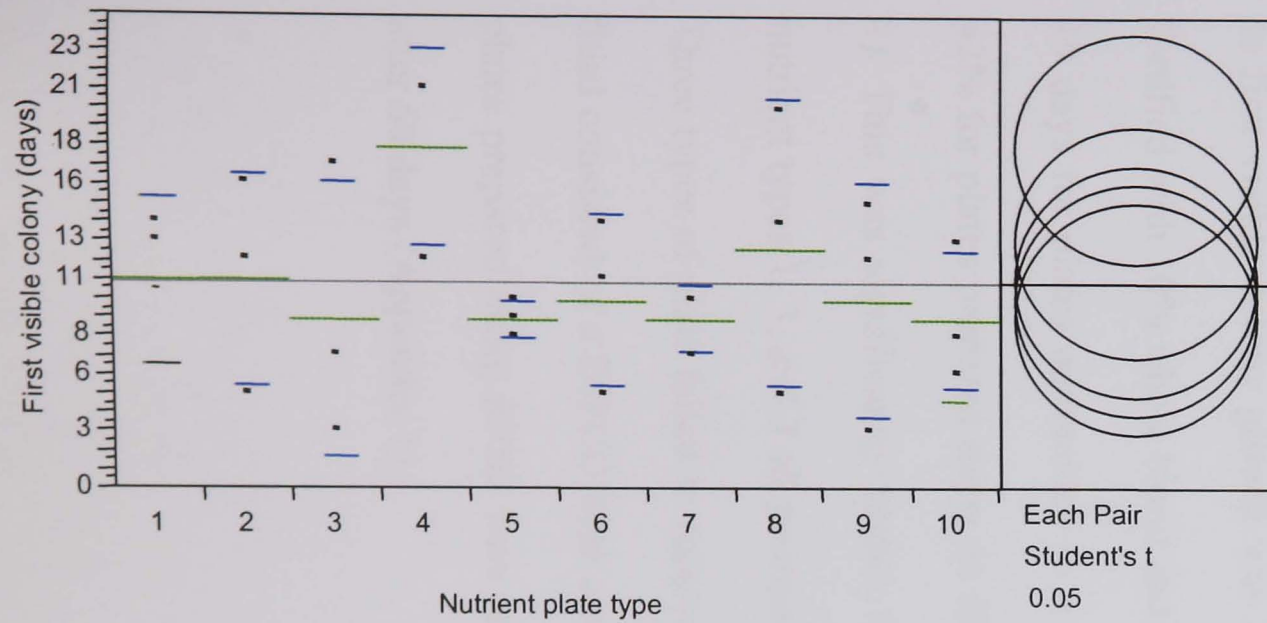
### 3.5.2 Growth of strain X10/1

When maintained in a 4% CO<sub>2</sub> all plate types supported growth of strain X10/1 (TC1) (Table 3.2, Figures 16a-d). There was no statistically significant difference between the type of nutrient plate used and time to first colony growth (Figure 16a).



However certain trends were noticeable. Time to first visible colony growth was 9 days, using four different plate combinations (nutrient types 3, 5, 7 and 10, Table 3.2, Figure 16a). These all contained a DIFCO base. The plate combination supporting the significantly highest plating efficiency (Figure 16b) after 60 days (75%), and the highest number of viable colonies after 60 days (70%, Appendix 1), were plates prepared using DIFCO base with 10% whole blood and with RPMI overlay (type 7). Plates supporting the lowest plating efficiencies were those containing DIFCO base fortified with 10% FCS (Figure 16b, nutrient types 4 and 8). All harvested colonies were successfully expanded at 30 days.

When maintained in sealed plates all plate types supported growth of strain X10/1 (TC1) (Table 3.2, Figure 16c and d). There was no significant difference between nutrient plate types and time to first visible growth. However, certain trends were observable. Time to first visible colony growth was 18 days, using 3 plate types (1, 3 and 7). This was 9 days longer than the equivalent strain maintained in a 4%, CO<sub>2</sub> humidified incubator. The plate type supporting the highest plating efficiency (61%), including the highest number of viable colonies after 60 days (80%, Appendix 1), was plates prepared using DIFCO base with 10% whole blood and with RPMI overlay. Plates supporting significantly fewer colonies were those containing DIFCO base fortified with 10% FCS. Interestingly, and unlike plates maintained in a CO<sub>2</sub> incubator, nutrient type 9 (DIFCO, 10% lysed blood with RPMI overlay) supported only low plating efficiencies (14%). All harvested colonies were successfully expanded after 30 days. After 60 days 70% of colonies remained viable on plates with DIFCO base and with saline or RPMI overlays.



94 Figure – 16a, Strain X10/1 maintained in a 4% CO<sub>2</sub> incubator. Time to first visible colony on different nutrient plate types. Green horizontal lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test (see text for explanation)

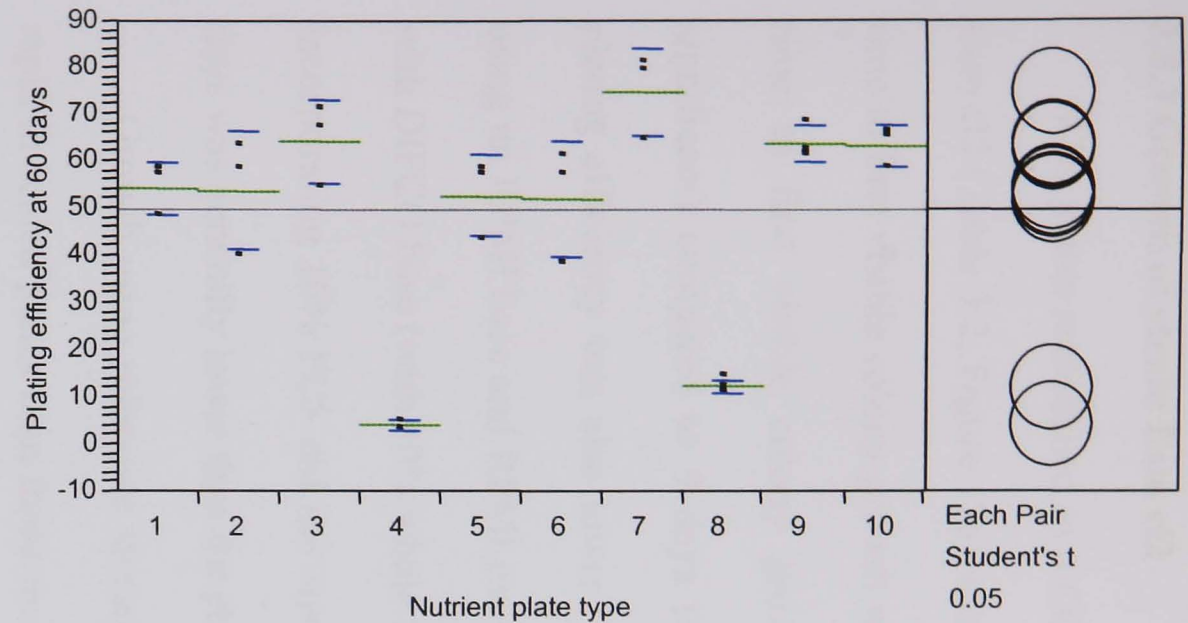


Figure – 16b, Strain X10/1 maintained in a 4% CO<sub>2</sub> incubator. Plating efficiency of different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test (see text for explanation)

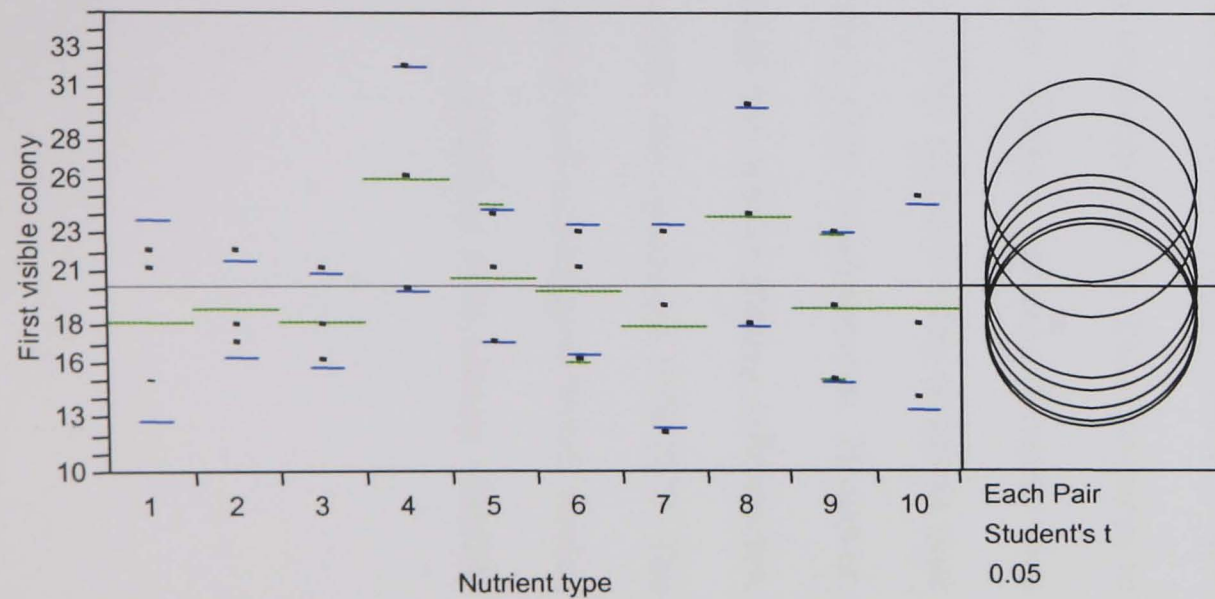


Figure – 16c, Strain X10/1 maintained in sealed plates. Time to first visible colony on different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test (see text for explanation)

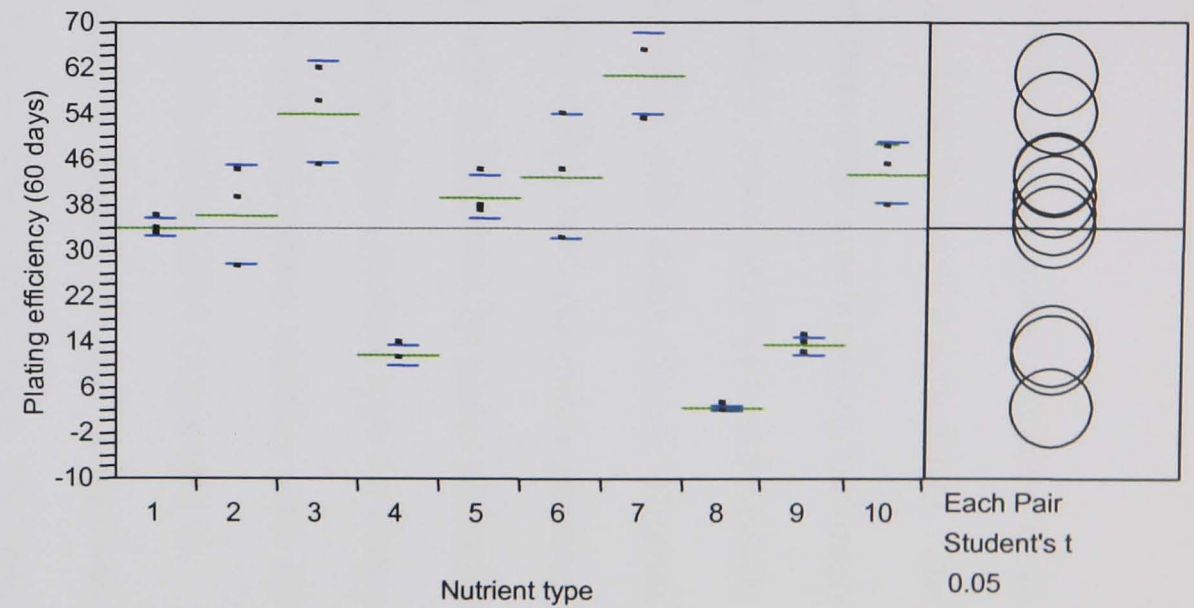


Figure – 16d, Strain X10/1 maintained sealed plates. Plating efficiency of different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test (see text for explanation)



### 3.5.3 Growth of strain Esm cl2

With plates maintained in a CO<sub>2</sub> incubator colony growth using reference strain Esm cl2 (Table 3.2, Figure 17a) was less rapid than those inoculated with X10/1. The time to first visible colony growth was 19 days on three plate combinations (although time to first visible colony growth between plate types was not statistically significant) compared to 9 days for plates inoculated with strain X10/1. Highest plating efficiency was also lower (Table 3.2, Figure 17b), 38% for plates prepared using an RPMI base and RPMI overlay (nutrient type 2) followed by plates prepared with DIFCO base (with 10% whole blood) and saline overlays (nutrient type 3). Plates incorporating 10% FCS did not sustain growth. In addition colony viability after 60 days was generally lower than for plates inoculated with strain X10/1 (Appendix 1).

Growth using reference strain Esm cl2 (Table 3.2, Figure 17c and d) was less rapid in sealed plates than those maintained in a CO<sub>2</sub> enriched atmosphere. The time to first visible colony growth was 21 days on plates prepared using a DIFCO base fortified with 10% whole blood and saline overlay (nutrient type 3). This compares to 19 days for plates maintained in a CO<sub>2</sub> atmosphere. Highest plating efficiency was 42% for plates prepared using an RPMI base and RPMI based overlay (nutrient type 1). This was significantly higher than for all other plate combinations. However, nutrient types 1, 3, and 7 all possessed relatively high but similar plating efficiencies. Three types of plate failed to support growth, of which two contained 10% FCS. The third consisted of a DIFCO base containing 10% lysed and centrifuged blood. Sealed plates prepared using RPMI base and RPMI overlay exhibited 90% colony viability after 60 days (Appendix 1).

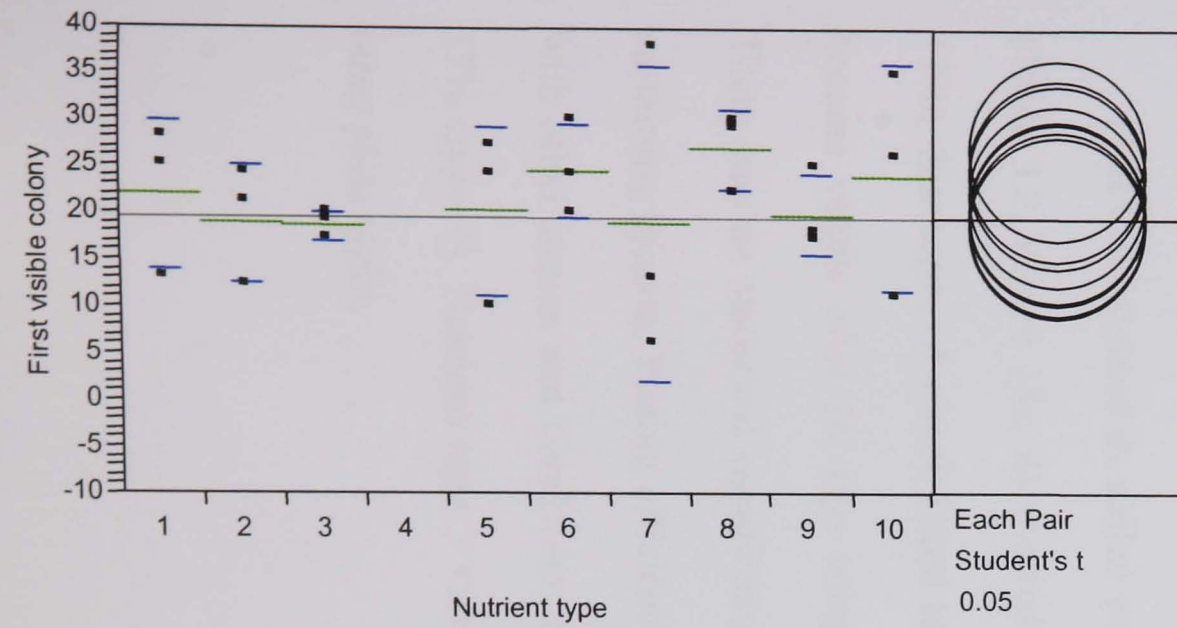


Figure – 17a, Strain Esmc12 maintained in a 4% CO<sub>2</sub> incubator. Time to first visible colony on different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.

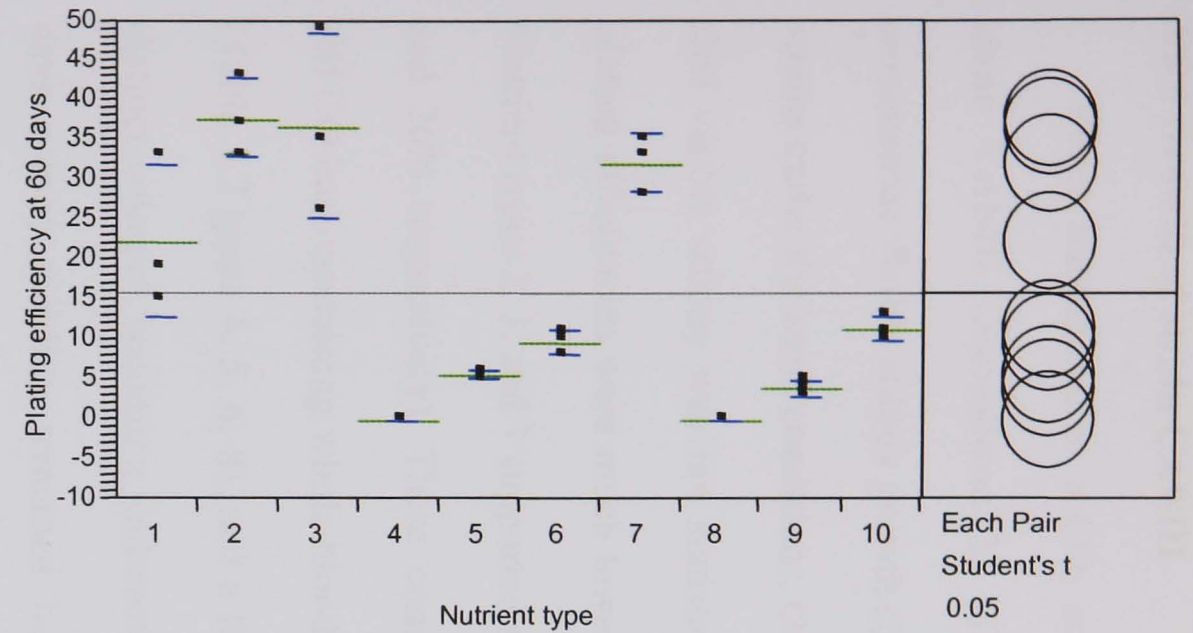


Figure – 17b, Strain Esmc12 maintained in a 4% CO<sub>2</sub> incubator. Plating efficiency of different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.

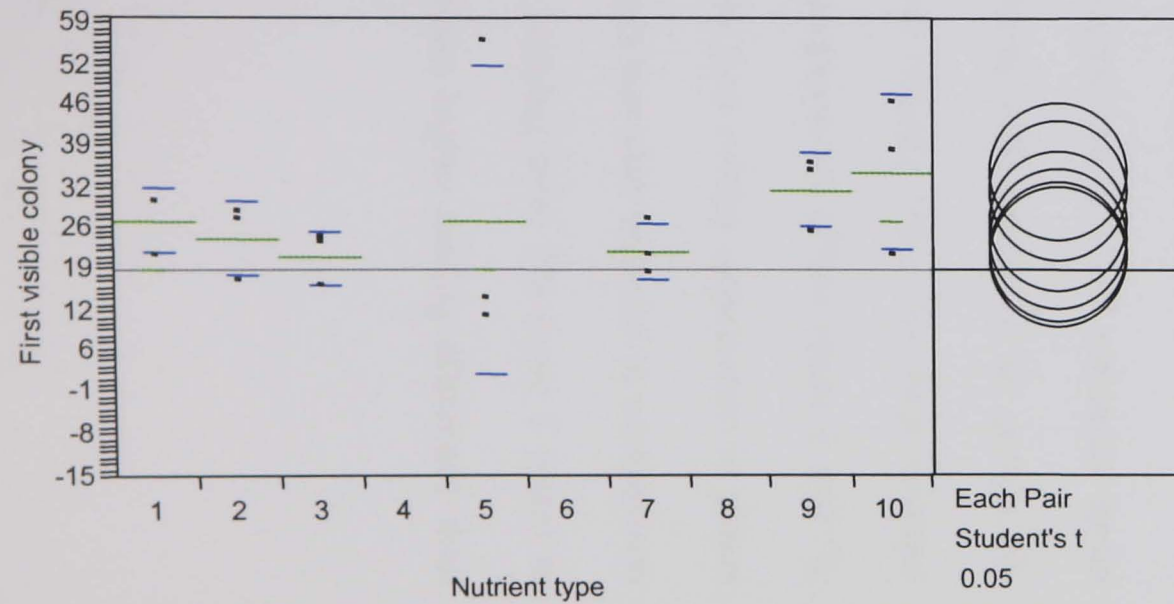


Figure – 17c, Strain Esmc12 maintained in sealed plates. Time to first visible colony on different nutrient plate types. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.

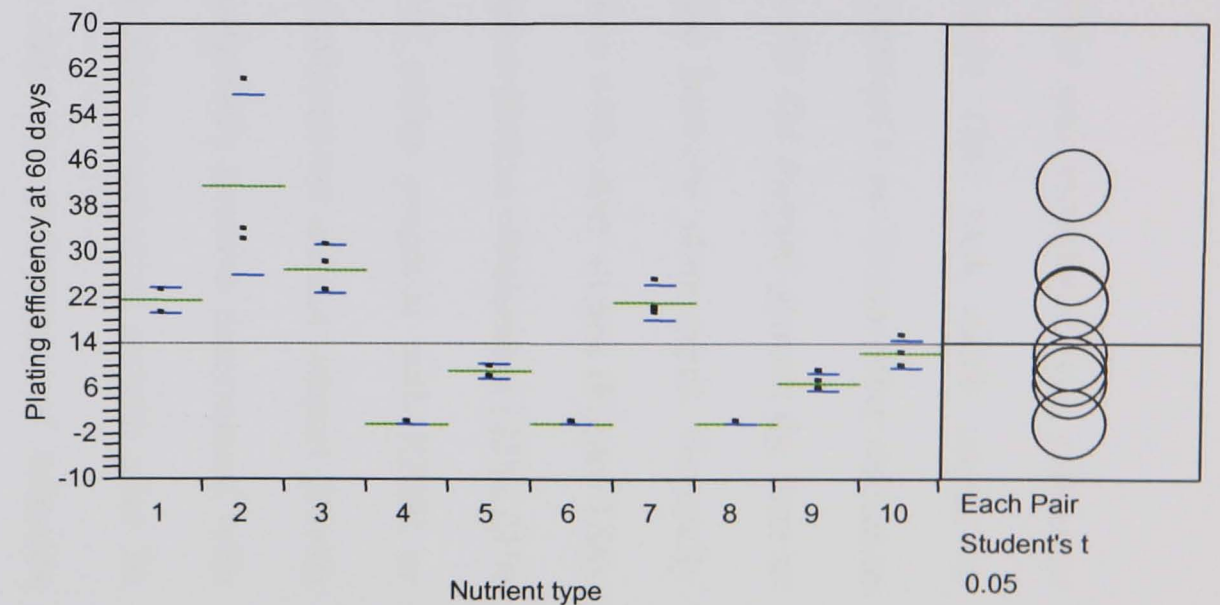


Figure – 17d, Strain Esmc12 maintained sealed plates. Plating efficiency of different nutrient plate types. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.

### 3.5.4 Growth of strain CANIII

Plates maintained in a CO<sub>2</sub> enriched incubator and inoculated with reference strain CANIII (Zymodeme 3) (Table 3.2, Figure 18a) took much longer to demonstrate visible colony growth (27 days using nutrient type 7) than other reference strains under the same conditions. On those plates that did support growth the time to first visible colony was not statistically significant between plate types. Generally plating efficiencies were much lower (0 - 26%) than with other strains (Figure 18b). Nutrient types 2, 3, and 7 supported statistically higher plating efficiencies (22%, 21% and 26% respectively). These consisted either of plates prepared with RPMI or DIFCO base containing whole blood. Four plate combinations did not support growth (Table 3.2 types 4, 5, 6, 8), and a fifth (type 9) originally positive deteriorated with distinct colonies becoming undetectable. In those plates supporting growth after 30 days colony viability remained high, after 60 days the percentage of colonies remaining viable was comparable with other reference strains (Appendix 1).

Plates maintained in sealed plates and inoculated with CANIII reference strain (Table 3.2, Figure 18c, d) showed the lowest plating efficiencies of all groups (0-17%). Six types of plates failed to sustain growth (Figure 18c). The first colonies became visible after 26 days using two plate combinations (Plate types 2 and 7). There was no statistical significance in the time to first colony appearance in plates sustaining growth. Plating efficiency (after 60 days) was also reduced in comparison with other strains and conditions, positive plates ranging from 2% (type 1 plate) to 17% (type 7). Nutrient type 3 sustained a significant higher plating efficiency than other plate types.



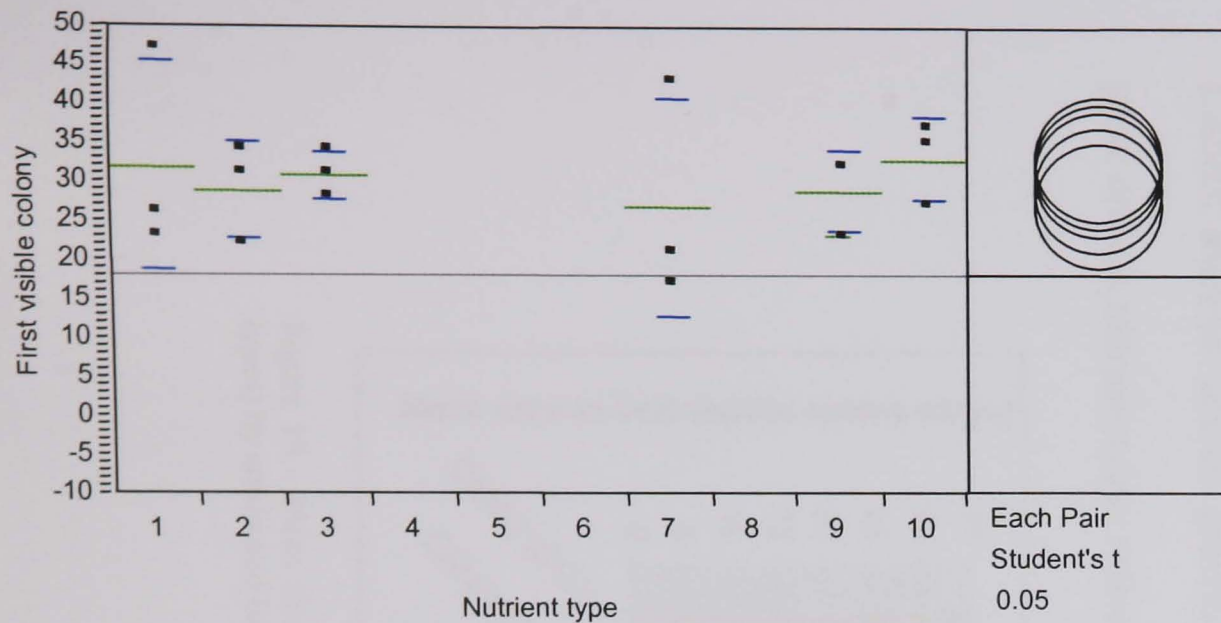


Figure – 18a, Strain CANIII maintained in a 4% CO<sub>2</sub> incubator. Time to first visible colony on different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.

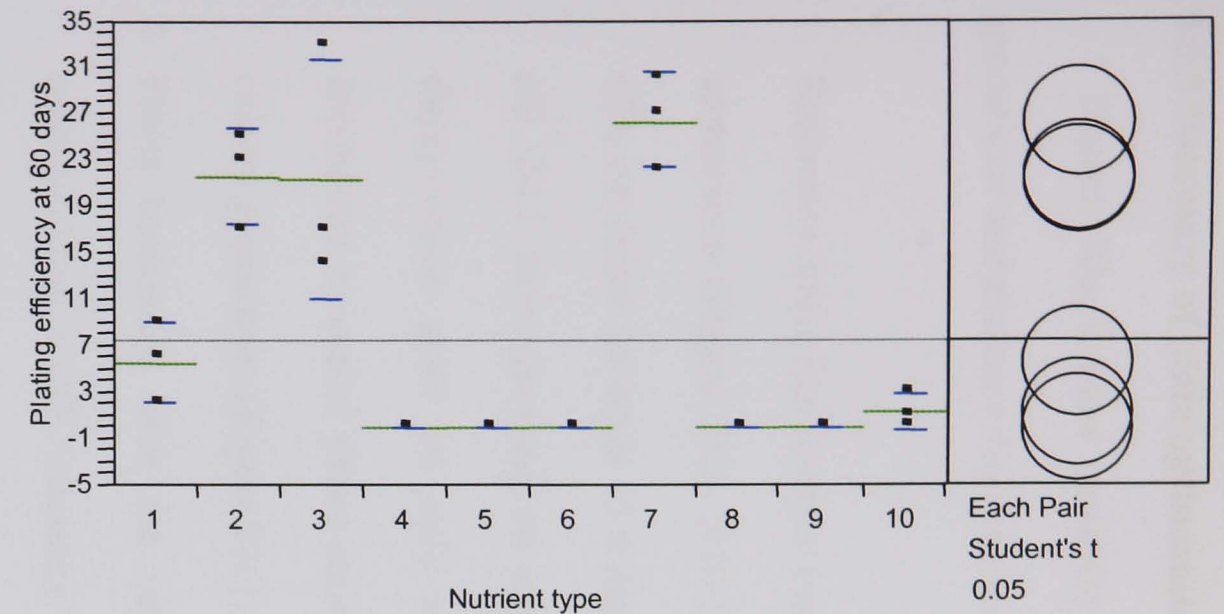


Figure – 18b, Strain CANIII maintained in a 4% CO<sub>2</sub> incubator. Plating efficiency of different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.

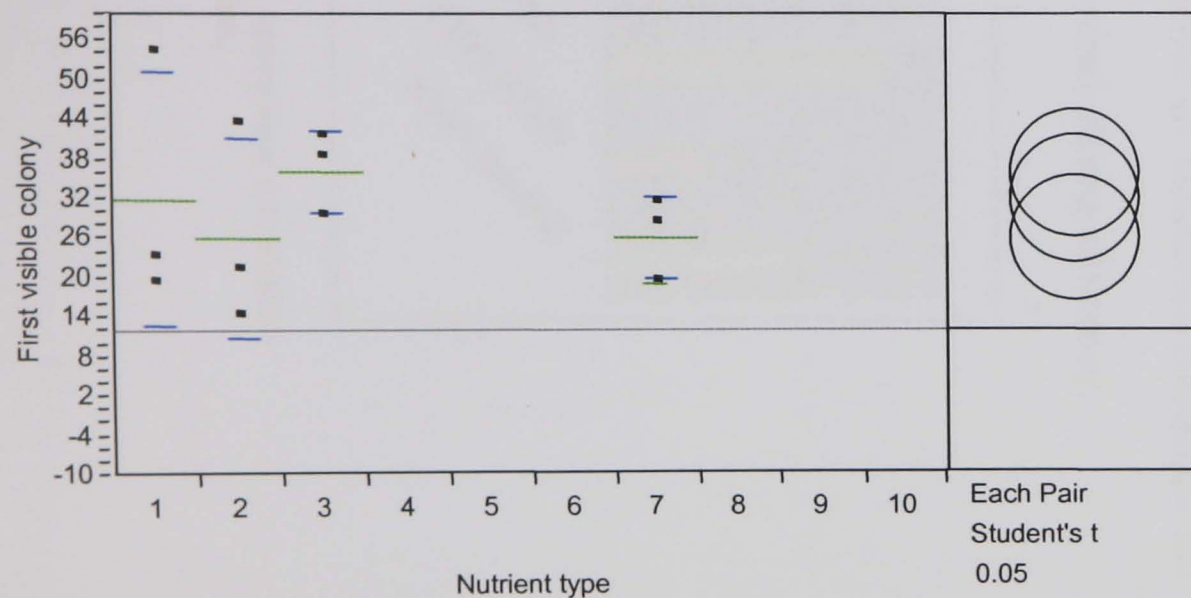


Figure – 18c, Strain CANIII maintained in sealed plates. Time to first visible colony on different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.

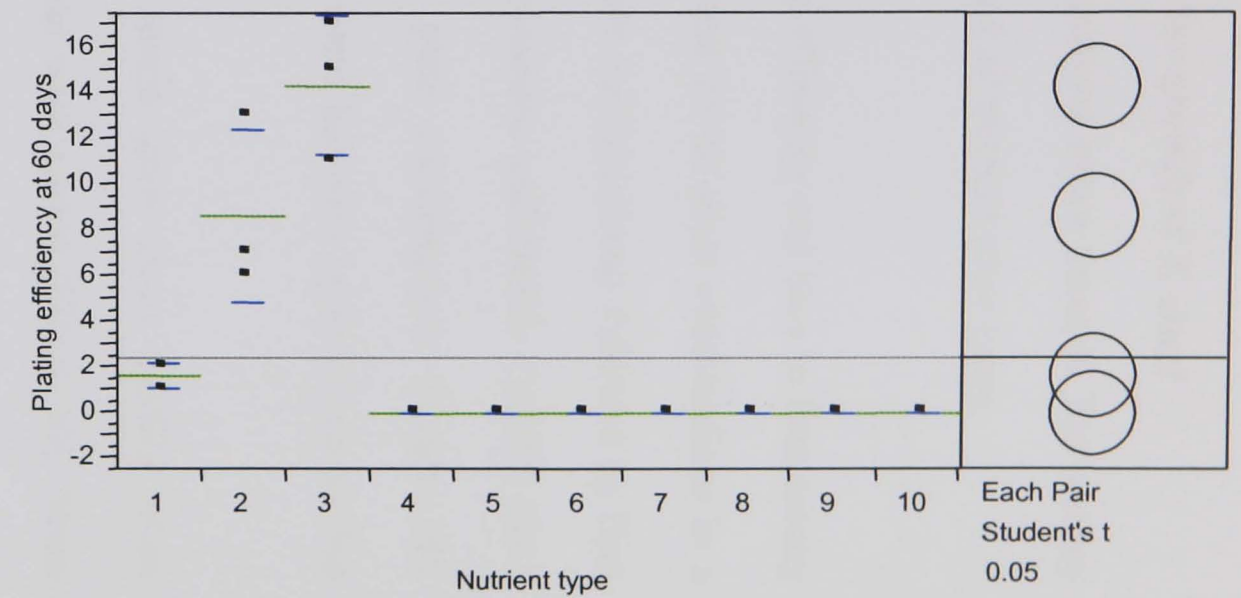


Figure – 18d, Strain CANIII maintained in sealed plates. Plating efficiency of different nutrient plate types. Horizontal green lines represent the group mean. Blue lines represent standard deviation from the group mean. Comparison circles plot show a visual representation of the Each Pair Student's t test.



3.5.5 Summary of plate optimisation conditions for growth of *T. cruzi*

Figures 20a, b show variability charts summarising mean times to first colony appearance and plating efficiency for each strain and all nutrient plate types.

1. Reference strain has a major impact on plating efficiency and time to first colony appearance (Figure 21a). X10/1 grew the fastest on all plate combinations in a CO<sub>2</sub> incubator (average 15.4 days using all plate combinations) followed by Esm cl2 (24.1 days) growing on some plate combinations and finally CANIII (30.1 days) which grew on only relatively few plate combinations (Figure 18). Incubation in sealed plates showed a similar trend but with greater time to first colony appearance (Figure 21c).
2. Plates inoculated with the same reference strain grew more quickly when maintained in a CO<sub>2</sub> incubator than plates that were sealed (Figure 19). Strain CANIII showed similar growth times irrespective of incubation conditions but grew on less nutrient types reflected in lower overall plating efficiency.

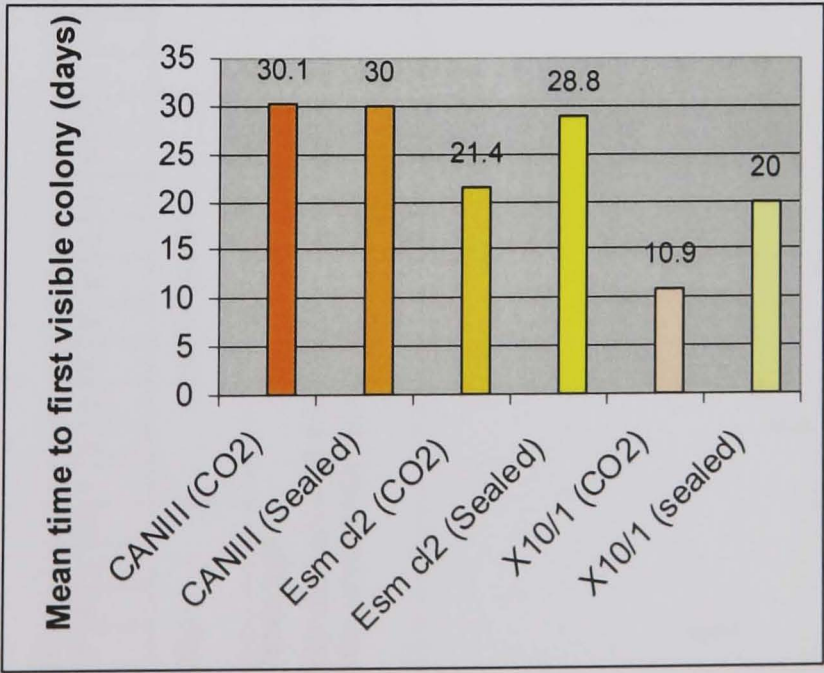
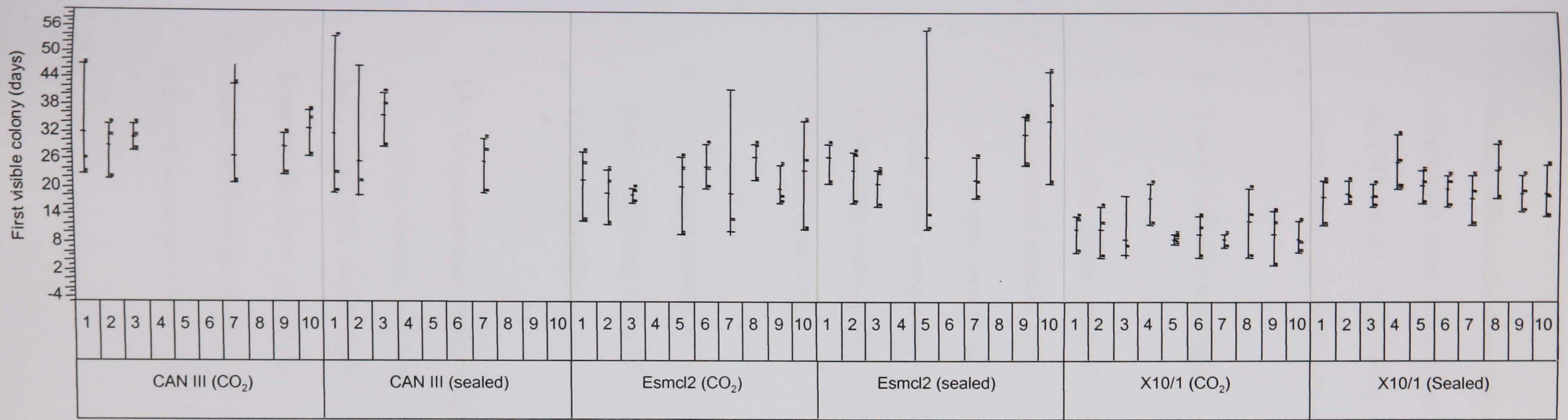


Figure 19 - Mean time to first colony appearance (all nutrient types) by strain and incubation conditions.



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Figure – 20a, Variability chart showing first visible colony (days) for each nutrient plate type, strain and incubation conditions. Vertical black lines on the Y axis join standard deviation points. Column with no data indicate that the nutrient type did not support growth.

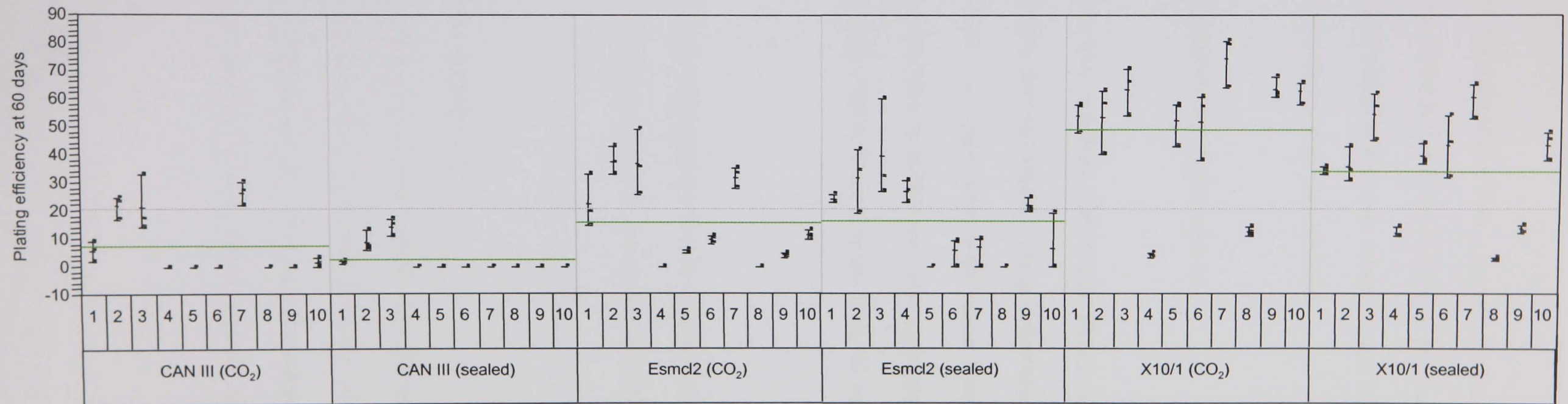


Figure – 20b, Variability chart showing plating efficiency at 60 days for each nutrient plate type (1 to 10), strain and incubation conditions. The horizontal green line represents the group mean. Vertical black lines on the Y axis join standard deviation points.



3. Plating efficiencies were highest in plates maintained in a CO<sub>2</sub> incubator. Highest efficiencies were achieved with reference strain X10/1 (75%) followed by Esm cl2 (61%) and CANIII (26%). Efficiencies in sealed plates were lower. Figure 21b shows plating efficiencies for each strain as a mean of all nutrient types.
4. Colony viability was higher at 30 days than at 60 days (Appendix 1).
5. Generally plates consisting of a DIFCO base fortified with whole blood and saline based overlay sustained more colonies and had higher plating efficiencies over a longer period of time (Figure 21c). The same base with saline based overlay gave comparable results. Plates containing an RPMI base also supported high colony viability and numbers, particularly with reference strains Esm cl2 and CANIII (Figure 21c).
6. Plates incorporating 10% FCS (types 4 and 8) supported the fewest colonies with plates containing lysed blood intermediate between the two. As shown in Table 3.2 and Figure 21c.

### **3.5.6 Trypanosomes isolated from laboratory infected mice**

Varying volumes of whole blood and plasma were incorporated into DIFCO plates with 10% whole blood and RPMI overlay. One batch of 10 plates was discarded due to a contaminated supply of rabbit blood used in plate preparation. Mixed infections inoculated into mice 21 days previously were used to provide the source of organisms (Table 3.3).



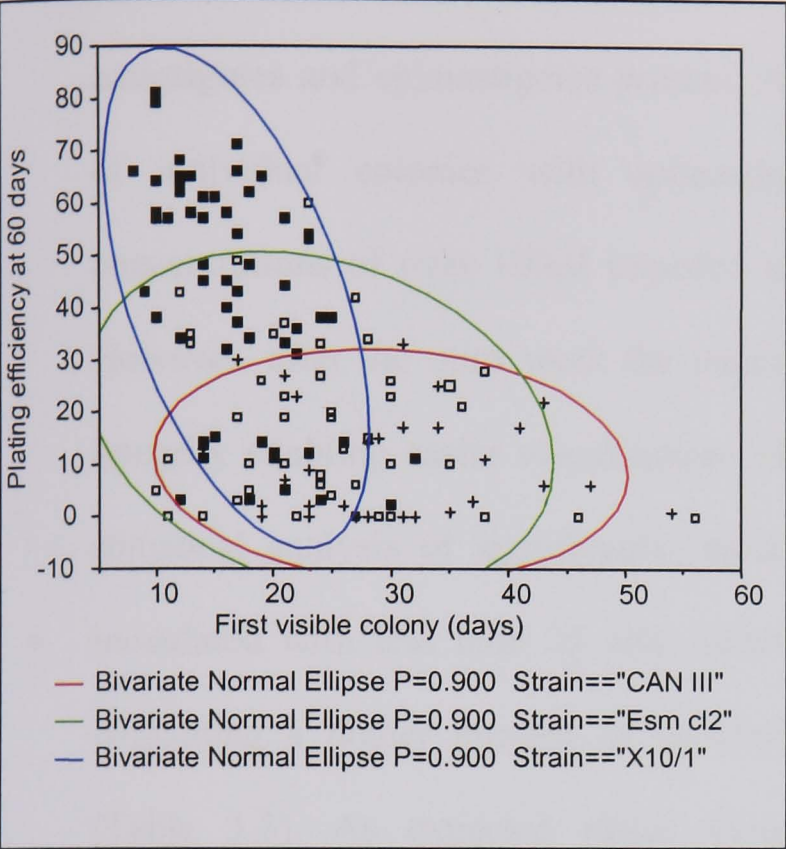


Figure 21a - Bivariate fit (comparison of strain types) of plating efficiency at 60 days by first visible colony. All nutrient types and incubation conditions.

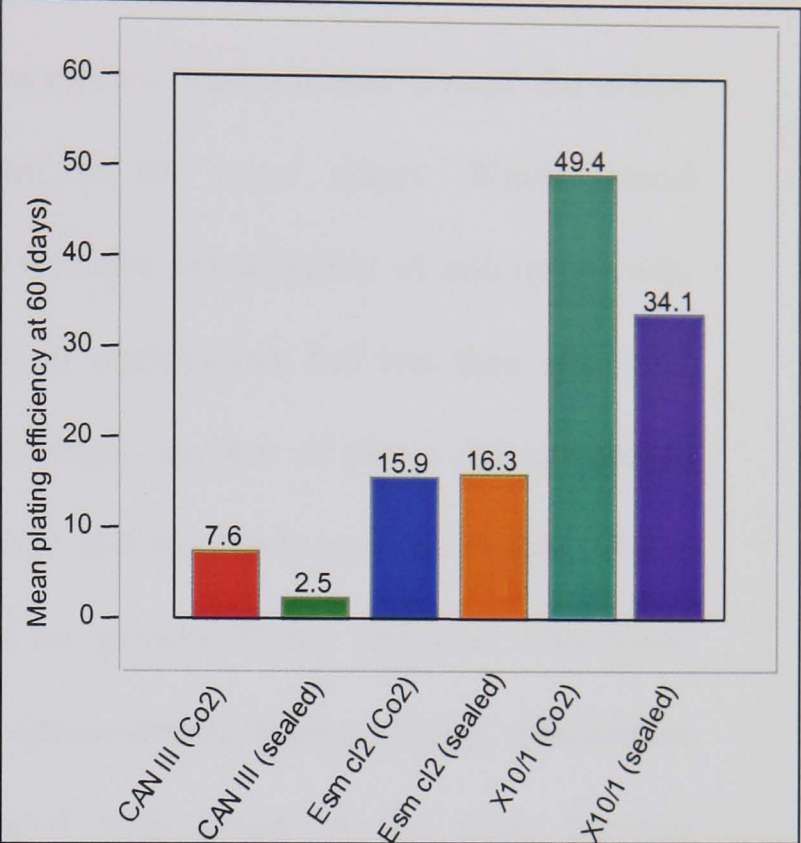


Figure 21b - Comparison of strain plating efficiency by incubation conditions. All nutrient combinations.

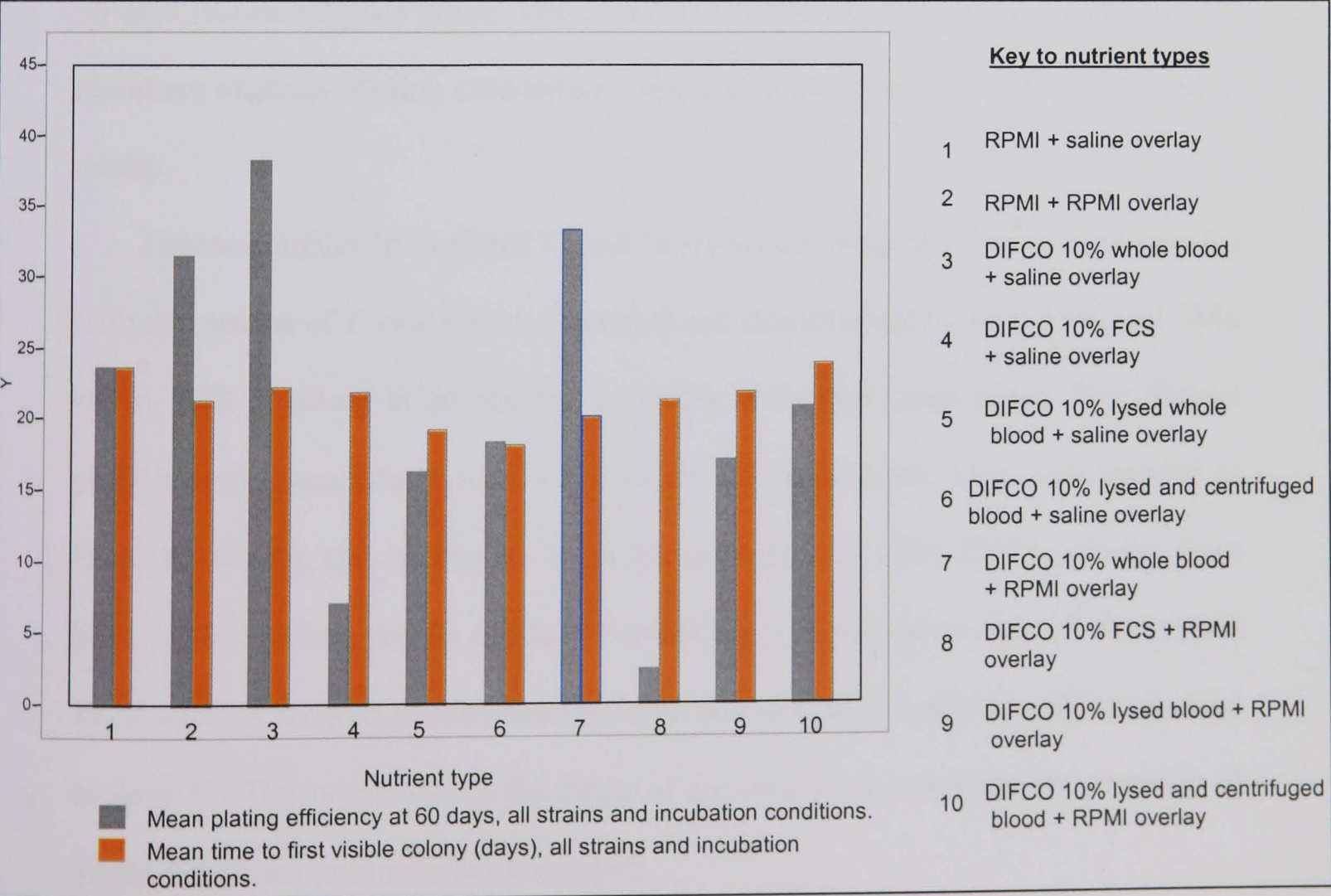


Figure 21c – Mean plating efficiency at 60 days and mean time to first visible colony (days). Mean values include all strain types and incubation conditions.

Five of 14 plates showed some growth. Colonies became visible at between 26 and 35 days. Colonies were typically 0.2–1.5mm in size after 35 days with amastigotes and epimastigotes present. Amastigotes predominated toward the centre of individual colonies with epimastigotes at the outer edges. Whole blood concentrations of over 100µl impeded microscopic visualisation of colony growth. However, after the third week the majority of erythrocytes had lost their structural integrity enabling easier visualisation. The limited number of plates did not enable statistical analysis of significance. However, certain trends are observable. Plates inoculated with less than 25 cells showed no growth. Plates prepared with blood containing a higher number of parasites demonstrated higher plating efficiencies (Table 3.3). As expected plates inoculated with blood of the same parasite concentration but using larger volumes demonstrated higher plating efficiencies. After 38 days (across all plate types) 73% of colonies were successfully expanded into 1ml of culture medium. Plating efficiencies using whole blood or plasma produced similar results.

Fifteen colonies from plates 13 and 14 (prepared using blood from mice infected with two strains of *T. cruzi*) were expanded and characterised by mini-exon and 24Sα rRNA PCR profiling in an attempt to resolve individual strain types. Two distinct profiles were successfully resolved from mixed inoculations. One corresponded to Esm cl2 (TC2b), and another to Arma 12. (Figures 22a, 22b). Of 15 colonies from plate 13, 8 showed a profile similar to Esm cl2 and 7 identical to Arm 12. From plate 14, 3 colonies showed an identical profile to that of Esm cl2 and 12 profiles identical to Arm 12. This result shows the value of the method in resolving the presence of mixed infections from infected mammals.





Figure 22a – Mini exon profile of clonal colonies obtained from a mixed infection in a single mouse. Lanes 2 and 3 show original inocula reference strains (TC2b and Arma 12 respectively). Lanes 4 -7 show clonal colonies identical to ARMA 12 profiles. Lanes 8 – 11 show clonal isolate profiles identical to TC2b profiles. No single colony has a mixed profile.

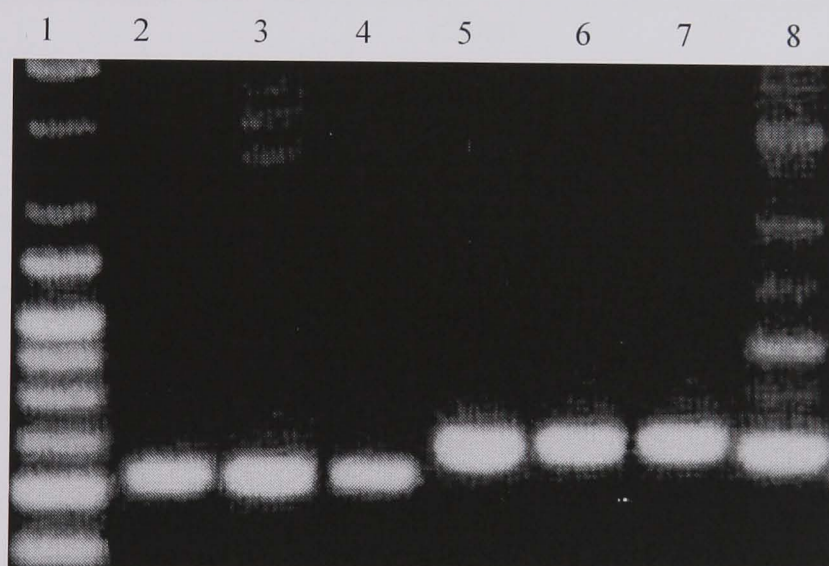


Figure 22b – 24S $\alpha$  rRNA profile of clonal colonies obtained from a mixed infection in a single mouse. Lanes 2 and 8 show original inocula reference strains (Arma 12 and Esm cl2 respectively). Lanes 3-4 show clonal colonies identical to ARMA 12 profiles. Lanes 5–7 show clonal isolate profiles with TC2b profiles. No single colony has a mixed profile.

### 3.5.7 Plating *T. cruzi* isolated from sylvatic mammals

Attempts were made to obtain clonal colonies from 36 sylvatic mammals (Table 3.4). Isolates were incorporated into either supplemented RPMI based plates or DIFCO based plates. At the time of plating thick film slides were prepared in parallel

Table 3.3 Plating trypanosomes isolated from experimentally infected mice.

Plate number	Inoculation strain	Time to first visible colony (days)	Inoculum type	Parasitemia (cells/ml)	No cells plated	plating efficiency at 30 days
1	Arm12 <sup>a</sup>	33	25µl	50	1.25	0%
2	Arm 12 <sup>a</sup>	28	50µl	50	2.5	0%
3	Arm 12 <sup>b</sup>	26	100µl	50	5	0%
4	Arm 12 <sup>a</sup>	32	100µl	50	5	0%
5	Arm 12 <sup>b</sup>	35	150µl	50	7.5	0%
6	Arm 12 <sup>a</sup>	25	150µl	50	7.5	0%
7	Arm12 + Esm cl2 <sup>a</sup>	26	25µl	250	6.25	0%
8	Arm12 + Esm cl2 <sup>a</sup>	32	50µl	250	12.5	0%
9	Arm12 + Esm cl2 <sup>a</sup>	25	100µl	250	25	8%
10	Arm12 + Esm cl2 <sup>a</sup>	32	10µl	6000	60	8.3%
11	Arm12 + Esm cl2 <sup>a</sup>	30	20µl	6000	120	10.8%
12	Arm12 + Esm cl2 <sup>a</sup>	28	30µl	6000	180	0%
13	Arm12 + Esm cl2 <sup>b</sup>	32	50µl	6000	300	6.6%
14	Arm 12 +Esm cl2 <sup>a</sup>	33	100µl	6000	600	24%

All plates were maintained at 28°C in a humidified CO<sub>2</sub> incubator, <sup>a</sup>denotes use of whole blood inoculum, <sup>b</sup>denotes use of plasma inoculum.

suggesting 7 of the collected mammals were positive for *T. cruzi*. This number was corroborated by subsequent dissection of triatomine bugs from xenodiagnosed mammals. Two methods were used in attempting the propagation of clones. Initially the triple centrifugation concentration technique was performed. Using this method no growth was visible on plates even after two months. Cell counts revealed infected animals had an extremely low parasitaemia (maximum 25 cells/ml) equivalent to 3.7 cells per 150µl. inoculation. The concentration method did not appear to increase appreciably parasite numbers in the sediment. Interestingly 14 animals (consisting of *D. novemcinctus* and *T. matacus*) infected with unidentified filarial worm larvae, did show a large increase in concentration of filariae following the triple centrifugation technique

Table 3.4 Mammals used in direct plating experiments.

<b>Mammal species</b>	<b>Number captured</b>	<b>Positive for <i>T. cruzi</i></b>
<i>Dasypus novemcinctus</i>	7	3
<i>Euphractus sexinctus</i>	5	3
<i>Didelphis albiventris</i>	2	0
<i>Tolypeutes matacus</i>	11	0
<i>Monodelphis domestica</i>	3	1
<i>Chaetophractus</i> spp.	6	0
Bat (unidentified)	2	0

Mammals were deemed positive after microscopic examination of thick film slide preparations and by examination of the dissected gut contents of xenodiagnosed bugs. All mammals were collected from sylvatic or peridomestic areas in Paraguay.

It was also apparent that the volume of supernatant which could be obtained after each centrifugation stage reduced dramatically. This resulted in the loss of a significant number of parasites discarded with the resultant sediments.

Similarly 60 plates prepared from the whole blood of 36 sylvatic mammals failed to show growth after two months. In addition biphasic blood slopes inoculated with 40ul of whole blood were negative. From this it appears that xenodiagnosis (using *T. infestans*) was the most sensitive technique for demonstrating the presence of *T. cruzi* infections in animals with low parasitaemia in Paraguay.

**3.5.8 Characterisation of clonal colonies isolated from five specimens of *R. robustus*: isoenzyme analysis**

Reference strains used represent the three principal zymodemes X10/1 (TC1), Esm cl2 (TC2), CANIII (Z3) clearly distinguished the three enzyme systems ASAT, PGM, GPI, Figure 22c. Selected isolates of interest (defined as those yielding clones

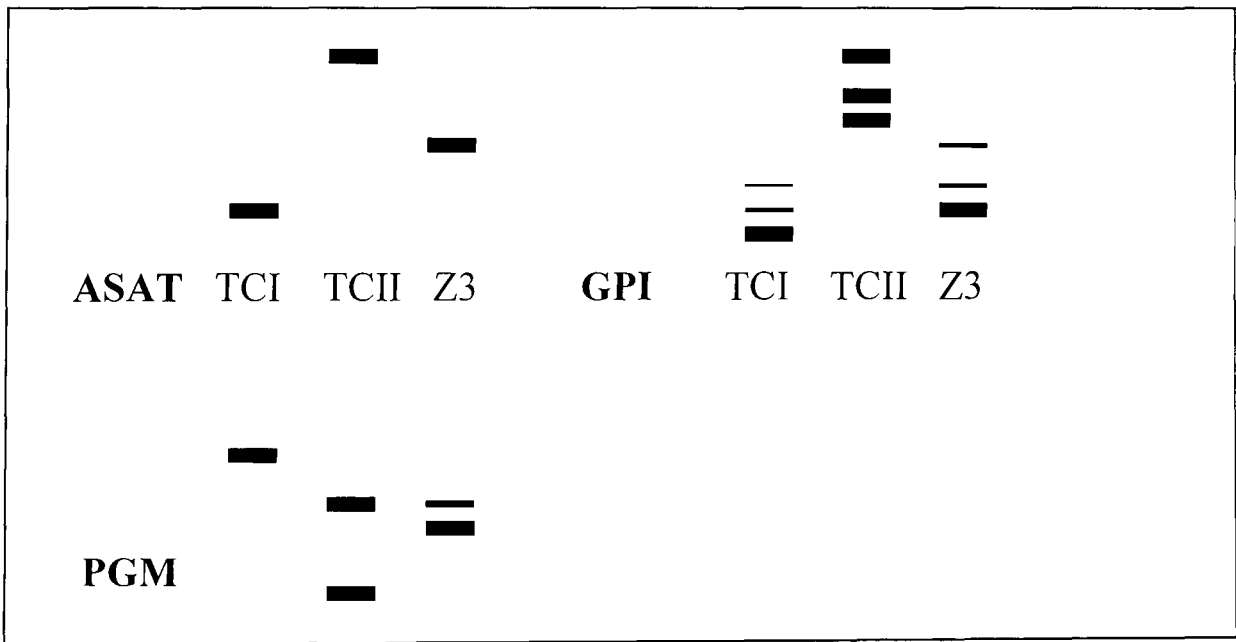


Figure 22c - Diagrammatic representation of isoenzyme profiles of the three principal zymodemes.

with different RAPD profiles, 3.5.9) were analysed with ASAT, PGM, GPI and also 6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase

(G6PD), which have been previously demonstrated to resolve the reference strains (Chapman *et al.*, 1984).

Twenty clonal colonies from each triatomine bug showed identical migration patterns with the ASAT and PGM enzyme systems. Figures 23A shows 14 such clonal colonies using the ASAT enzyme system and 23B shows 5 clonal colonies using the PGM enzyme system. Thus colonies from each of the individual bugs gave identical monomorphic migration patterns, all corresponding to TC1 reference strain X10/1.

Using the GPI enzyme system (Figure 23C) all colonies isolated from bugs 304, 78, 83 and 84 corresponded with reference strain X10/1 (TCI). All colonies isolated from individual bugs gave identical migration patterns. However profiles of *T. cruzi* colonies isolated from bug 303 could be clearly distinguished from those isolated from bug 304, 78, 83 and 84. Isolates from bug 303 showed a triple GPI band with three equidistant bands and a central band that was more intense than the outer bands suggesting a heterozygous GPI locus.

### **3.5.9 RAPD analysis of *T. cruzi* stocks**

Isolates obtained from individual bugs were characterised using 8 primers (A1, A2, A3, A4, A6, H1, L4, L5; Table 2.1, Chapter 2). Primer A3 produced bands of a lower visual quality than other primers and was discarded from the analysis. The remaining primers produced a clear correspondence with the TC1 reference strain. RAPD profile, examples of which are shown in Figures 25 and 26, and are in agreement with isoenzyme profiles.

Profiles generated from all primers (except primer A6) produced identical profiles from 20 clonal isolates from individual bugs. Profiles generated by



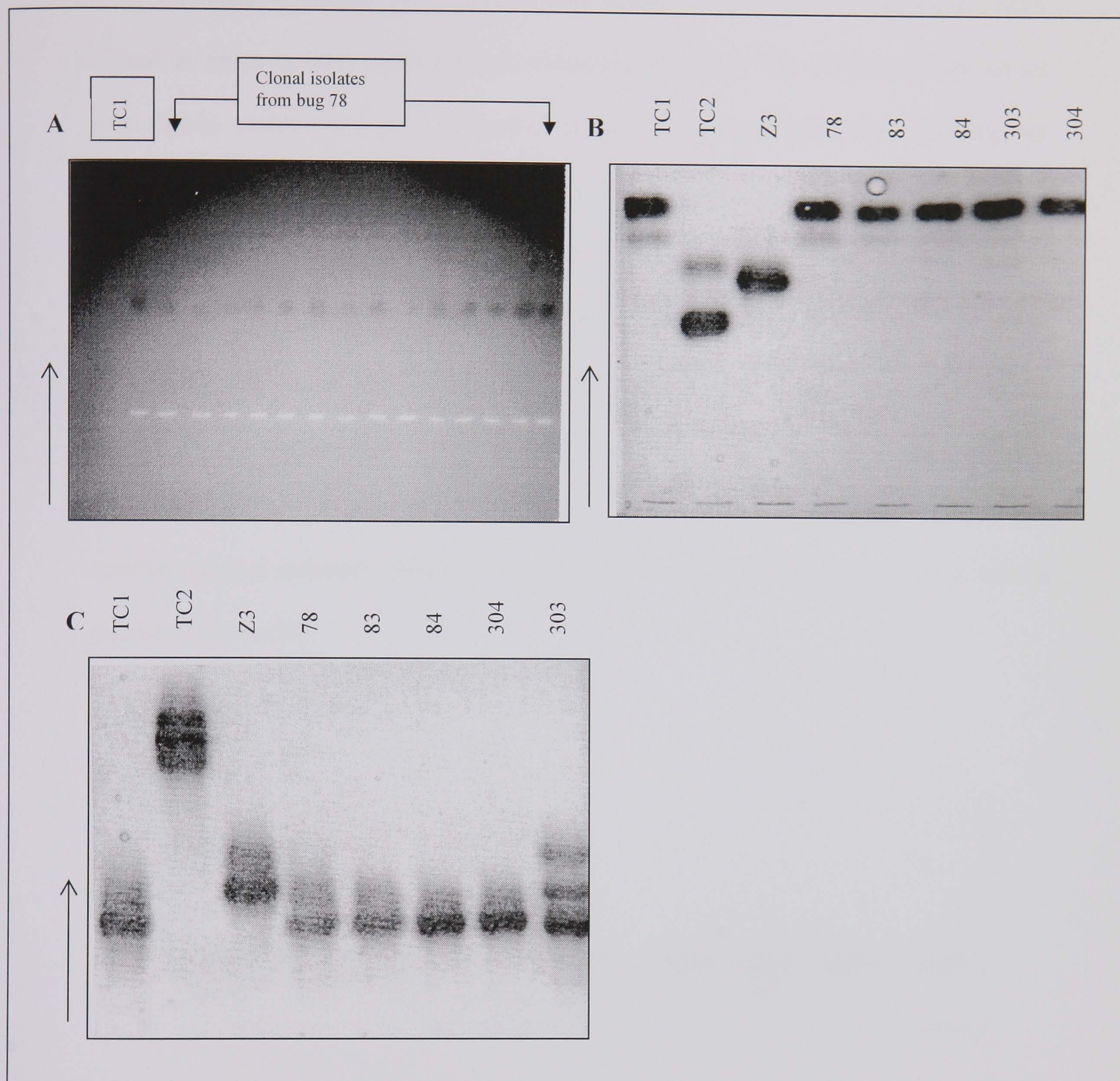


Figure - 23 Isoenzyme profiles for *T. cruzi* isolated from triatomine bugs. A, ASAT profiles of clonal isolates showing identical migration profile. B, PGM isoenzyme analysis of *T. cruzi* extracted from different triatomine bugs. C, GPI profile of *T. cruzi* from different bugs. Isolates from bug 303 produced a triple banded profile.

primers A4 (Figure 24) and H1 were able to distinguish between isolates from bugs 303 and 304 compared to isolates from bugs 78, 83 and 84. Primers A2 and L5 could distinguish isolates from bug 303 from bugs 304, 78, 83, 84 (Figures 25 and 26 respectively).



Importantly, primer A6 revealed two distinct RAPD profiles obtained from clonal colonies isolated from a single triatomine bug (bug 78). Of 20 colonies tested one colony produced a profile distinct from the others (Figure 27). The original cryopreserved isolate (stored directly following bug dissection) was re-plated and a further 20 colonies analysed with primer A6. Three colonies produced profiles distinct from the remaining seventeen. Profiles matched those of the previous run. The most likely explanation for the difference in RAPD profiles is the presence of a mixed infection. Additional isoenzyme systems 6-phosphogluconate dehydrogenase (6-PGD) and glucose 6-phosphate dehydrogenase (G6PD) were applied to the same resultant clonal colonies. Profiles corresponded to TC1 with no variation in clonal profiles (Figure 28).

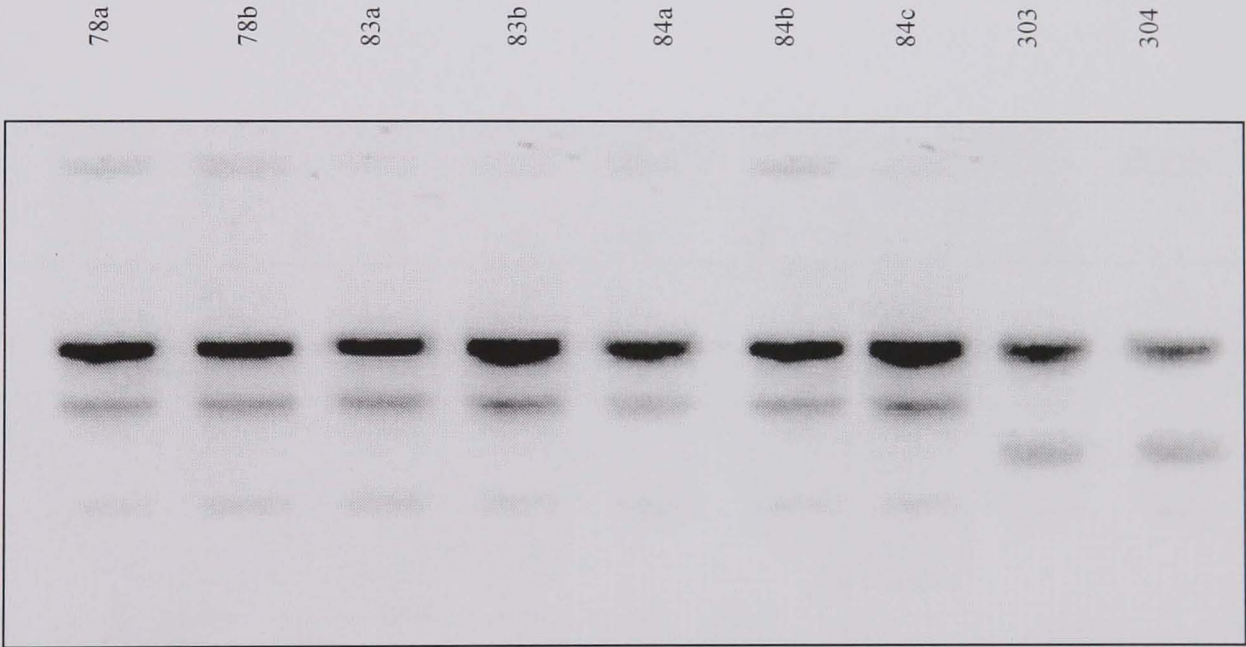


Figure 24 - RAPD profile from bug isolates generated using primer A4, distinguishing isolates from bug 303/304 from those of bug 78, 83, and 84.



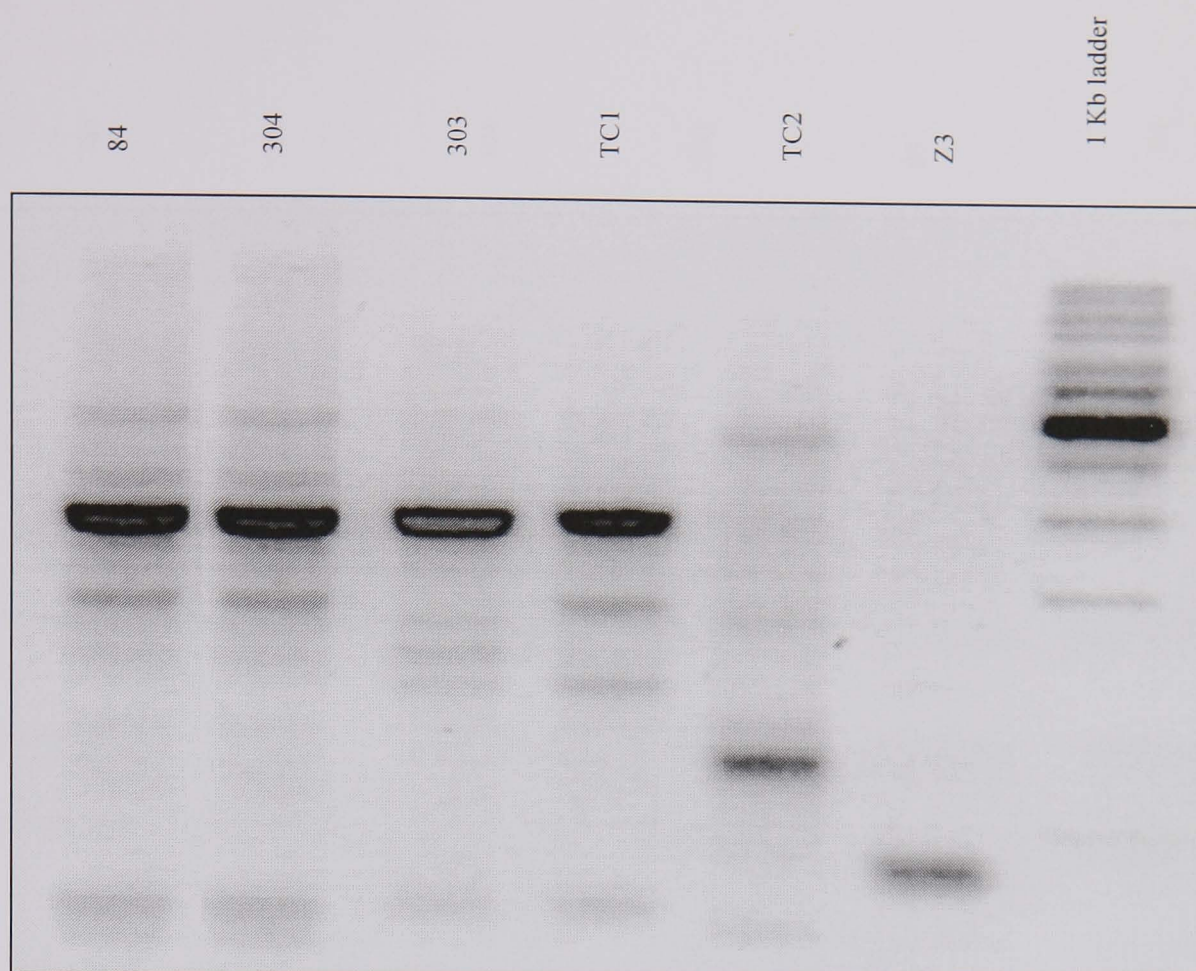


Figure 25 - RAPD profile from bug isolates generated using primer A2, distinguishing isolates from bug 303 from those of bug 304 and 84.

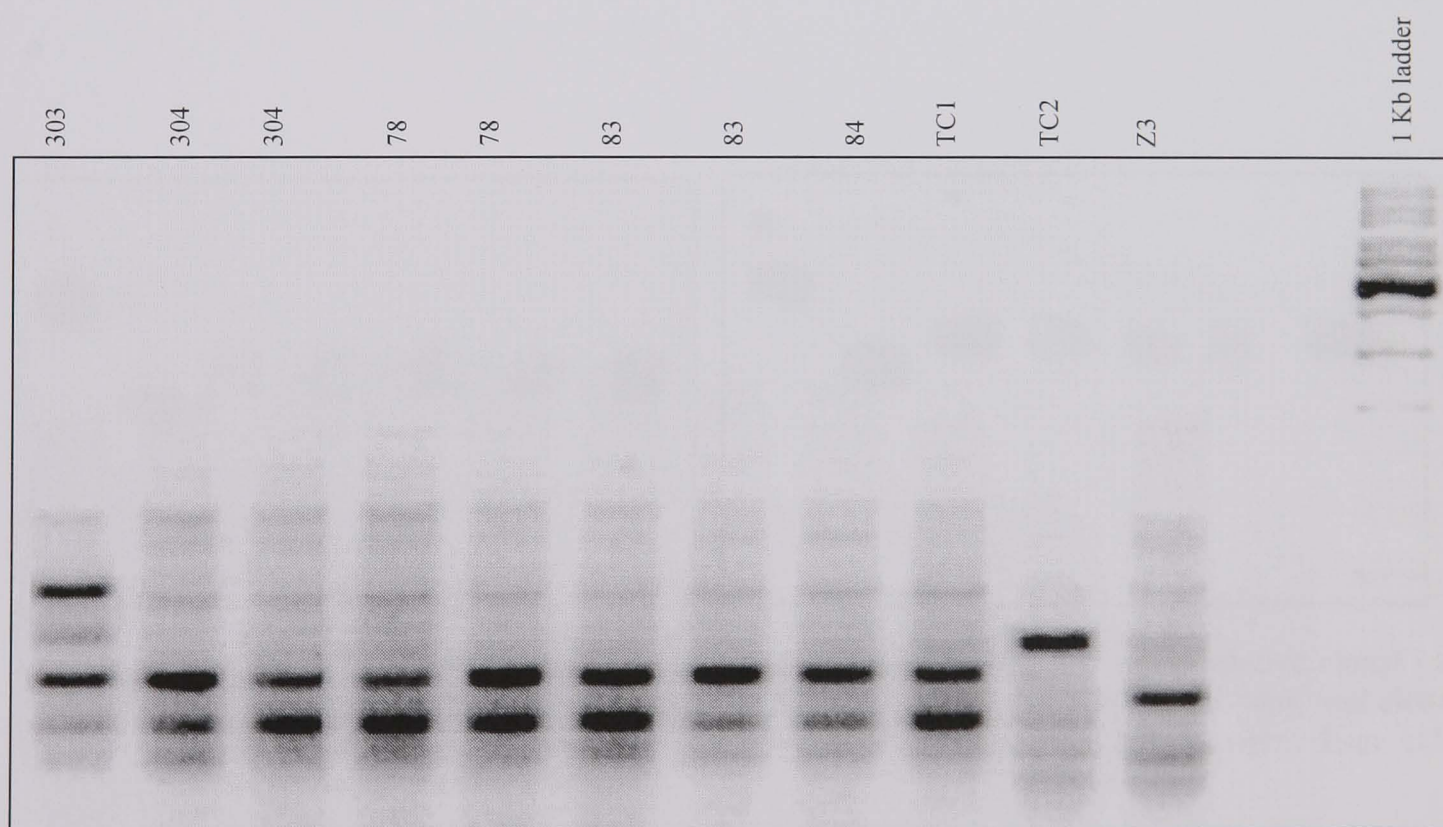


Figure 26 - RAPD profile produced with primer L5 distinguishing isolate 303 from other bug isolates.



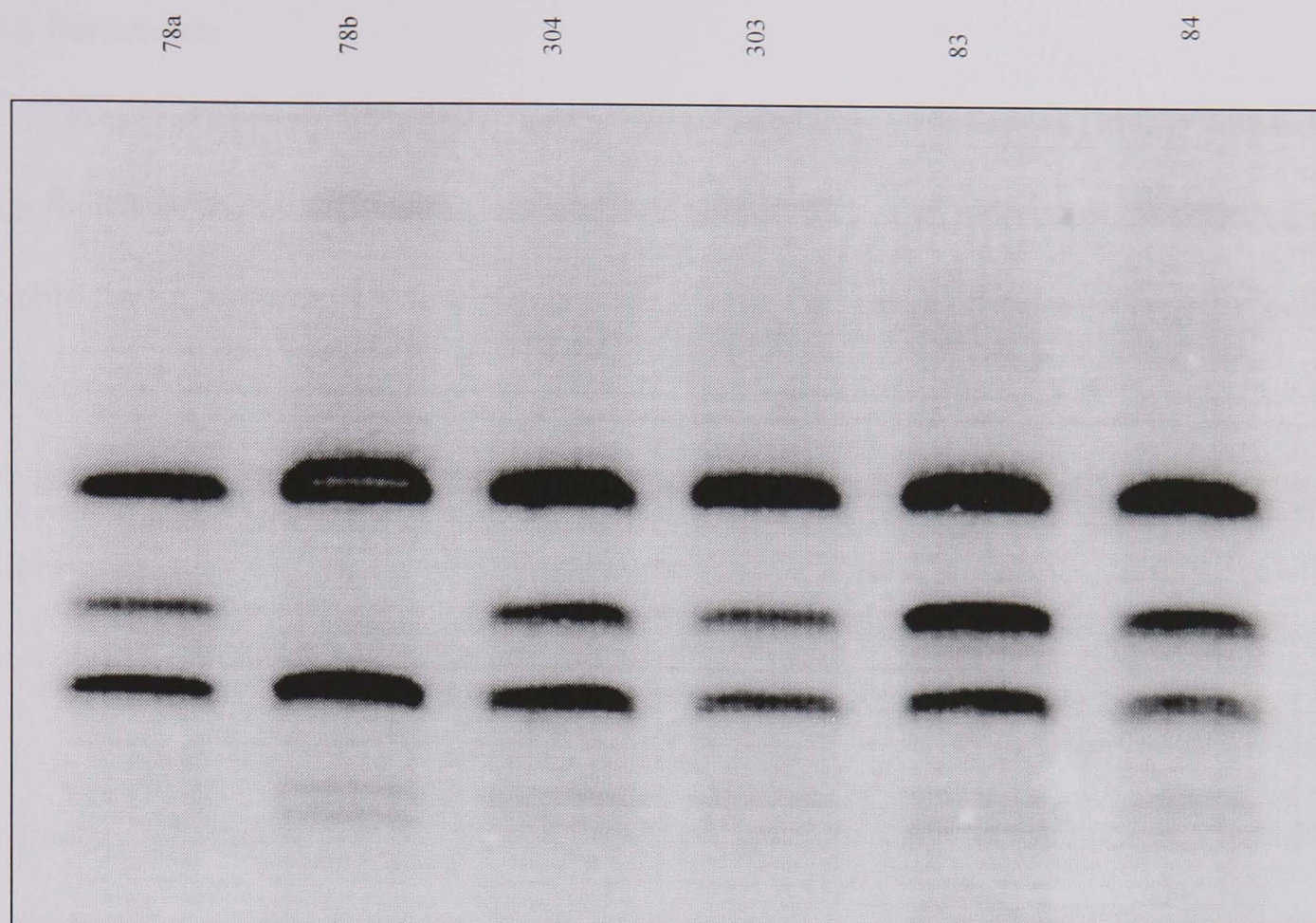


Figure 27 - RAPD profile using primer A6 showing a mixed infection in two clonal colonies isolated from bug 78 (ladder not shown).

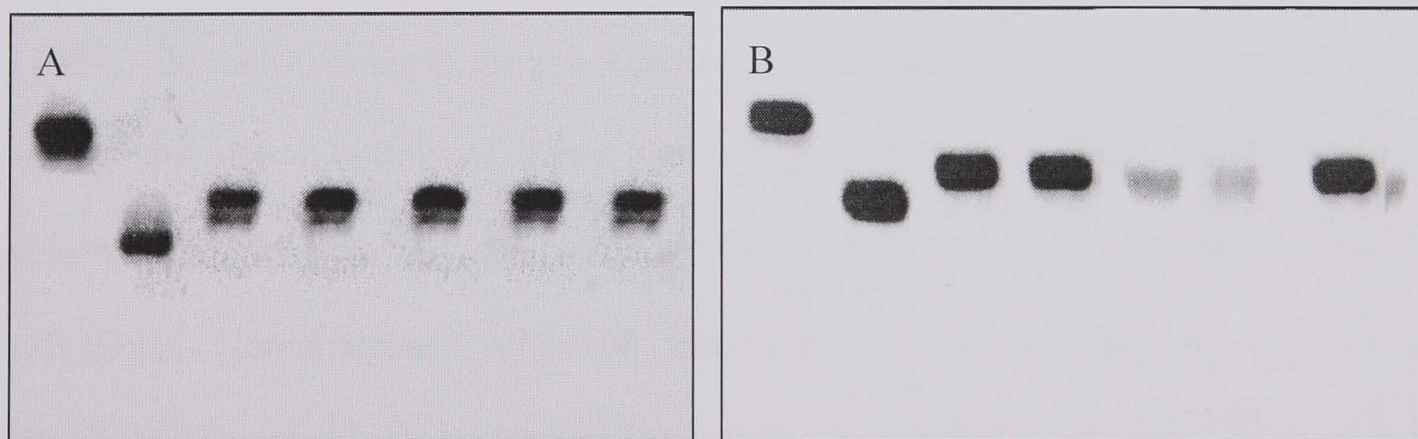


Figure 28 - A, Isoenzyme profile (6PGD) showing four representative clonal isolates from bug 78. All profiles correspond to TC1. B, Isoenzyme profile (G6PD) showing four clonal isolates from bug 78. All correspond to TC1. Order of loading from left to right: Esm cl2, CANIII, TC1, representative clones.

### 3.6 Discussion

Previous cloning techniques have resulted in plating efficiencies from cultures in the region of 40%, with colony isolation possible in 21 days, and colonies remaining viable for up to 40 days (Mondragon *et al.*, 1999). Our results demonstrate significant improvements, with viable colony isolation possible in as little as 9 days, maximum plating efficiencies of 75% and colony viability at over 60 days. However this was highly dependent on strain type. Furthermore the technique allows for colony growth in a non CO<sub>2</sub> incubator.

Large differences were apparent in the time to first colony appearance. The primary influencing factors were strain type, nutrient plate combinations, and the subsequent incubation conditions. Reference strain TC1 showed prolific growth on most plate combinations in comparison to CANIII that grew on only 4 of the 10 nutrient combinations used. The difference in growth rates had been previously noted during cultivation in liquid culture (Finley & Dvorak, 1993). Lauria-Pires *et al.* (1997) observed different growth kinetics and doubling times in different *T. cruzi* strains and also in biological clones grown in axenic liquid medium. The isolation of discrete colonies thus has obvious benefits, if hosts and bugs may possess mixed infections. Xenodiagnosis, although sensitive for detecting infected animals is unsuitable for resolving multiclonality due to the possibility of overgrowth of one genotype. The lack of a resolution of mixed infections has been previously reported to cause hindrance in molecular typing (Solari *et al.*, 1998).

Generally the combination of a DIFCO base containing 10% defibrinated rabbit blood with a saline overlay supported the fastest time to first colony appearance, highest number of colonies, and highest plating efficiencies with many colonies

remaining viable after 60 days. Similar results were obtained using RPMI as an overlay. The results are particularly relevant where clonal isolation of slow growing strains is concerned. The exceptions were reference strain Esm cl2, and CANIII (incubated in a 4% CO<sub>2</sub> atmosphere), which also grew well on plates incorporating an RPMI base and overlay.

The results also demonstrated that all strains tested could be cultured in sealed plates without the need of a CO<sub>2</sub> enriched atmosphere (although growth was noticeably less rapid on these plates). One possible explanation would be the time required for CO<sub>2</sub> to accumulate in sealed plates as a by-product of respiration, *T. cruzi* growth is inhibited by basic conditions (Mondragon, 1999). This observation is particularly relevant in many laboratories in South America where such CO<sub>2</sub> facilities are often unavailable. Previous publications had reported desiccation to be a primary factor in reducing colony viability. Colonies on many plate combinations remained viable after 60 days, although viability was reduced. The increased viability, in comparison to that of Mondragon *et al* (1999) is possibly the result of the use of a thick basal layer of agar forming a larger moisture reservoir in comparison with those prepared using LMP alone. Incubator humidification is crucial, sealed plates have the advantage of reducing the prospect of desiccation further, accounting for higher colony viabilities in comparison with plates closed with porous tape to enable gaseous exchange. Poor growth on plates incorporating FCS was unsurprising as these plates have little haemin, utilized by *T. cruzi* as an iron source. Addition of haemin may aid clonal propagation. The various combinations incorporating lysed blood and lysed and centrifuged blood were attempts to enable increase visual resolution of colonies. Prior to the experiment it was hypothesised lysis would free cellular nutrients accelerating growth. This was not the case. It appears that in the initial growth on

plates, nutrient availability is not the limiting factor. In fact plates prepared using an RPMI overlay (and DIFCO underlay) showed marginally less growth than those possessing a saline overlay (not statistically significant). Visualisation of colonies on plates incorporating whole blood is more time consuming. Despite this, individual cells and developing colonies can be observed. After three weeks the loss of integrity of erythrocytes further aided visualisation. Colonies of 0.2mm were viable enabling isolation in as little as 9 days. It would, however, be entirely feasible for colonies to be visualised and microscopically removed much earlier. In this instance RPMI based plates, for optimum transparency, would be most suitable. Twelve plates out of a total of 180 became contaminated. This low rate of contamination is expected as reference inocula originated from sterile culture.

Using isolates plated directly from triatomine bugs the contamination rate was much higher (35% of plates). It was noticeable that the majority of contamination was fungal. Improvements could involve validation of higher concentrations of antifungal agents in the growth media and White's Solution. The identification of a mixed infection in a single triatomine bug by RAPD analysis implies this is a frequent occurrence as only 5 specimens were available at the onset of this study. Variations in isoenzyme profiles were not detected with the enzyme systems used. This is perhaps to be expected as variation within TC1 is less marked than within TC2 (Brisse *et al.*, 2001). Furthermore RAPD analysis provides a higher prospective resolution as a consequence of whole genome profiling rather than detecting variations in enzyme phenotypes.

Attempts to isolate clones from sylvatic mammals in South America were unsuccessful using triple centrifugation and whole blood inoculations. Seven animals were shown to be positive by xenodiagnosis. The low parasitaemia was the most



likely cause of failure. (typically 25 cells/ml or 3.7 cells per 150µl inoculation). The triple centrifugation method was largely inefficient, with many parasites lost with discarded sediment. Whole blood and plasma inoculation using blood with low parasitaemia is also not likely to be successful. This is in accordance with Wittner *et al.* (1982) who observed that inoculations of less than 100 resulted in low plating efficiencies. The mechanisms are not well understood, cells may communicate with each other through the secretion or excretion of soluble factors. In contrast, Santos *et al.* (2000) reported no effect on sensitivity or growth by the addition of preconditioned growth media to agarose plates. Depletion of glucose has been implicated in stimulating differentiation (Tyler & Engman, 2000) but has not been shown to be a trigger for accelerated growth. Therefore, it appears that a primary obstacle for obtaining clones from mammals is parasite burden. Experimental results from inoculated mice corroborate this. Clonal colonies were successfully propagated with a minimum inoculum of 25 parasites (using cultured strains). It was also apparent that plating efficiencies (maximum 24%) were lower than those used in the culture experiment. It is likely this is a result of parasite death due to the presence of complement in the mouse blood during differentiation. The susceptibility of *T. cruzi* epimastigotes to lysis by normal or immune sera in a complement-dependent reaction has been reported (Fernández-Presas *et al.*, 2001). In this work we suggest that optimum efficiency for plating from mammals involves identifying individuals with high parasite burdens and using maximum inocula. The triple centrifuge technique showed minimal efficacy. The use of whole blood and low centrifugation to settle erythrocytes followed by 2 hr incubation at 37°C was as effective.

Success in isolating clones from sylvatic animals could be resolved by the use of immunosuppressants with the prospect of increasing parasite burdens. Additionally

anion exchange separation (Lanham's column) will be undertaken in future analysis (4.7.3).

This study shows that clonal isolation of multiple infections from single vectors and mammal hosts is feasible. Plating efficiencies from mammal isolates are possible given high parasite inocula. The method has been demonstrated to work well for triatomines in 3 laboratories in Brazil and also in Paraguay, where our collaborators now use the technique. This study provided an essential methodological approach to enable analysis of field isolates of *T. cruzi* (Chapter 4.0).

## **4.0 CHARACTERISATION OF DOMESTIC AND SYLVATIC ISOLATES OF *T. cruzi* IN PARAGUAY.**

### **4.1 Paraguay: geography, climate and population**

Bordered by Bolivia, Brazil and Argentina, Paraguay is a landlocked country positioned in relative obscurity in the heart of South America. Geographically it is situated between 19° 18', 27° 36' South and between 54° 19', 62° 38' West. Three major rivers influence the ecology, industry and social demographics (the rivers, Paraguay, Parana and the Pilcomayo). The river Paraguay dominates, dividing the country into two, the vast semi arid Chaco to the West and the more fertile subtropical environment situated to the East (Figure 29). Temperature fluctuations are common and range from an average of 14.5°C in winter to 31.5°C in summer. In the Chaco midday temperatures often exceed 45°C.

Paraguay is a relatively under-populated country with approximately 4 million inhabitants recorded in the 1992 census, and a crude birth rate of 4.9 children per 1000 (1980-85, Rojas de Arias, 1996). The mortality for infants under 5 was 42/1000 live newborn rising to 60.7/1000 in rural areas. Fifty seven percent of the population is situated in rural areas with 76.8% of this sector employed in the agricultural industry (Rojas de Arias, 1996). Half of all houses are considered inadequate with respect to physical structure and lack of basic services. In rural areas the most common construction material for walls is wood (38.2%) followed by brick (25.8%) and mud and wattle (25.8%). Fifty five percent of houses are constructed with straw roofs, 24.3% have tiled roofs with 11.4% constructed of wood. Sixty eight percent of dwellings have soil floors with 16% possessing brick floors (Consejo, 1991).





Base 801508 (541393) 5-90

Source: Lonely Planet

Figure 29 - Study areas in Paraguay. Departments of Boqueron, San Pedro and Paraguari are marked.



#### **4.1.1 Chagas disease in Paraguay**

The first confirmed case of Chagas disease in Paraguay was identified in 1939. The agent was isolated from a soldier from Fortin Linares in the Chaco region. By 1959, 55 cases were diagnosed with 72% of these cases occurring in the Western Chaco (Velazquez & Gonzalez, 1959). *T. infestans* was implicated as the primary vector. In Paraguay various studies have reported up to 14% of houses infested with triatomines and a prevalence of 20% in endemic areas (Rojas de Arias, 1996). Serological data from 679 Indians in the northern Argentina and western Paraguay (the Gran Chaco) demonstrated a 37.7% prevalence of *T. cruzi* infection (Basombrio *et al.*, 1999). Seropositivity increased with age and clustered in families. Seroprevalence varied between the 16 communities examined. A recent serological study encompassing all regions in Paraguay observed a 3.9% prevalence with one million people exposed to the risk of infection (Rojas de Arias, personal communication). Presently 300,000 people are estimated to be infected and an estimation of the incidence of the disease suggest 14,680 new cases each year in the absence of control (Rojas de Arias, 1996). This equates to an economic loss due to mortality and chronic Chagas of \$30 million per year.

#### **4.1.2 Control of Chagas disease in Paraguay**

Two basic principles are acknowledged for the prevention of Chagas disease in Paraguay. The first is the use of insecticides targeted at domestic and peridomestic triatomine foci. The second is establishing a community surveillance programme (Schofield & Dias, 1991). An ancillary aim is to prevent vector domiciliation by improving housing conditions. These factors are encompassed by Paraguay's participation in the southern cone initiative. Nevertheless, as in other Latin countries

the problem remains significant due to the lack of political impetus, and due to decentralisation and financial constraints. Despite this, more than 70,000 houses have been sprayed in endemic areas and wide coverage exists in transfusional control (Dias & Scofield, 1998). From the period 1983–1999 a 60% reduction in incidence is reported. An interruption in vectorial and transfusional transmission is expected by 2003 (Moncayo, 1999; WHO website: <http://www.who.int/ctd/chagas/epidemio.htm>).

#### **4.1.3 Vectors of Chagas disease in Paraguay**

Eleven species, from 3 genera have been described in Paraguay (*P. geniculatus*, *P. guentheri*, *P. lignarius*, *P. megistus*, *Triatoma delpontei*, *T. guayasana*, *T. guazu*, *T. infestans*, *T. platensis*, *T. sordida* and *P. coreodes*). The pattern of domiciliation mirrors that of other southern cone countries. The principal vector of Chagas disease is *T. infestans*, which has both active and passive dispersal behaviour. This species is considered to have originated in the Cochabamba valley in Bolivia as previously this was the only region where true sylvatic foci have been confirmed (Dujardin *et al.*, 1987). However, early reports indicate that specimens were occasionally found in sylvatic habitats in Argentina, Paraguay, and Brazil (Noireau *et al.*, 1997). The variety of ecotopes ranges from rocks, trunks of fallen trees and nests/burrows of marsupials and rodents. Doubt has been cast on the validity of some of these records in terms of misidentifications and the close proximity to dwellings. It was generally accepted that *T. infestans* did not maintain sylvatic foci. However, in 1997 four specimens of unidentified triatomine bugs were captured in the Bolivian Chaco (a continuation of the Paraguayan Chaco) 30km from the nearest human dwelling (Noireau *et al.*, 1997). Morphologically similar to *T. infestans* except for a darker coloration they were also superficially similar to the related and darkly pigmented species *T. melanosoma*. This



species is commonly found in peridomestic bird nests in Argentina and produces identical isoenzyme profiles to domestic *T. infestans*. Viable offspring were produced when the sylvatic *T. infestans* samples were crossed with domestic *T. infestans* under laboratory conditions. Due to these chromatic differences they were designated dark morphs (DM). Further studies (Noireau *et al.*, 2000) demonstrated the degree of differentiation of DM was clearly within the *T. infestans* intra-specific level. Nevertheless, marked chromatic and morphometric differences as well as differences in antennal pattern, chromosome banding and randomly amplified polymorphic DNA support the hypothesis of a distinct population. They concluded that continuous exchange of insects between wild and domestic habitats seems unlikely in the Bolivian Chaco but question the validity of the hypothesis that the Andean population represents the original wild focus (Schofield, 1988).

Important secondary vectors in Paraguay include *T. sordida* found in both sylvatic and domestic environments, *P. megistus*, *T. guyasana*, and *P. geniculatus*. All have epidemiological significance as they may invade or infest dwellings and peridomestic dwellings cleared of *T. infestans*.

#### **4.1.4 Strains of *T. cruzi* in Paraguay**

Limited studies have shown a remarkable genetic heterogeneity based primarily on isoenzyme phenotypes of domestic samples (Chapman *et al.*, 1984; Acosta *et al.*, 2001). Chapman (1984) concluded that the majority of the isolates in the domestic cycle were what is now known as TC2e, but also identified TC2c (e.g. strain X109). Acosta *et al.* (2001) demonstrated three distinct subgroups, two corresponding to Brazilian and Bolivian zymodemes with a third related to the Tulahuen stock, by characterisation using 13 enzyme systems. These equate to subgroups TC2b, TC2d

and TC2e respectively when using the classification parameters (discrete typing units, DTUs) defined by Barnabe *et al.* (2001). Thus all samples grouped within the major TC2 lineage. One isolate, derived from a sylvatic specimen of *Euphractes sexcinctus* (armadillo) also grouped within TC2. These findings corroborate those observed by Chapman *et al.* (1984) who found 90% of isolates possessed heterozygous isoenzyme profiles grouping within Bolivian zymodeme 2 (TC2). Three strains demonstrated homozygous isoenzyme profiles similar to those of the major Brazilian zymodemes. Thus heterozygous profiles predominate in the domestic transmission cycle in the Paraguayan Chaco. Prior to this PhD programme there are no studies on subgroup specific strain typing of sylvatic isolates in Paraguay.

#### 4.1.5 Reservoirs of Chagas disease in Paraguay

Few studies on *T. cruzi* reservoirs have been performed in Paraguay. Canese (1973, 1978) using a total of 17 wild animals found two specimens of *Didelphis albiventris* (opossum, Figure 30A) positive for *Trypanosoma* spp. The remaining animals consisting of two armadillo species *Dasypus novemcinctus* and *Tolypuetes matacus* (Figure 30B) were negative as was a single specimen of the fox *Cerdocyon thous*.



Figure 30 - A, *Didelphis albientris* a major reservoir of *T. cruzi*. B, *Tolypuetes matacus* widespread in the Paraguayan Chaco.

A further study (Fujita *et al.*, 1994) encompassing 112 domestic and 4 sylvatic animals (including one armadillo, *E. sexinctus*) from the Department of San Pedro revealed 24 positive domestic animals (of which 16 were dogs) but no positive sylvatic animals (tested by antibody to *T. cruzi* by direct agglutination).

Despite the lack of investigative studies in Paraguay, important inferences can be taken from findings in neighbouring countries. *D. albiventris* is one species with high infection rates (25-45%) in sylvatic ecotopes of Argentina and Brazil (Ramirez *et al.*, 2002). Here opossums play a crucial role as they can invade peridomestic areas providing a possible link between sylvatic and domestic transmission cycles. Similarly *D. novemcinctus* (the 9 banded armadillo) has been implicated as an important reservoir having a wide distribution throughout the American continent. This species presents infection rates of between 5.6-66.3% in Argentina, Brazil, Panama, Venezuela, French Guyana and Costa Rica (Carcavallo, 1999). The lack of resolution of sylvatic reservoir species and characterisation of their associated strains remains an important omission in understanding transmission cycles in Paraguay.

#### **4.2.0 Study locations**

Study locations were divided into three geographical regions; the Western Chaco within the Department of Boqueron (approximately 600km Northwest of Asunción), the central department of San Pedro (300km North of Asunción) and a final location 65km Northwest from Asuncion (Figure 29). A brief description of each of the sites follows.



**4.2.1 Boqueron in the western Chaco:** The western Chaco region is characterised by vast flat semi arid ecotypes. Vegetation is comprised of thorny scrub, palm tree forests in the South, and fine clay soil known as “Polvo” (Figure 31). Climactic vegetation is interrupted by vast estancia ranches, run by Germanic settlers (Mennonites). Settlements visited were located in areas surrounding the Mennonite towns of Filadelfia and Loma Plata (Figure 29, 31). These were:-



Figure 31 - Typical climactic vegetation of the northern chaco

*Campo Loro:* Situated 120km East of Loma Plata (Figure 34), the settlement consisted of approximately thirty low quality dwellings constructed of wooden walls with corrugated metal or wooden roofs, and soil floors (Figure 32). Inhabitants were indigenous Indians who were hunter gatherers, with occasional employment on local ranches.



*Santo Domingo*: 70km North of Filadelfia set in a typical semi arid environment. The village comprised approximately 25 low quality huts. Although primarily hunters, inhabitants also kept free roaming chickens and pigs.

*San Martin*: Geographically close to Santo Domingo the settlement consisted of 10 low quality huts. The inhabitants were not hunters but occasional work was performed on nearby Mennonite cattle ranches and dairy farms.

*Tiberia*: Consisted of 20 huts. In contrast to other sites some dwellings were constructed of wattle and daub as opposed to wood. Situated 80km from Filadelfia, their primary occupation was hunters with seasonal employment at local ranches.



Figure 32 – Typical Indian dwelling in the northern Chaco



#### 4.2.2 Department of San Pedro

San Pedro is a central department in Paraguay approximately 300km North from Asuncion (Figure 29); the department's capital is also called San Pedro and situated close to the river Paraguay. The ecology is more fertile (Figure 33) with much of the land utilised for agriculture and ranching. Across the river begins the southern-most point of the vast Paraguayan Chaco.



Figure 33 – Typical ecotype of the Southern Chaco and areas surrounding San Pedro.

#### 4.2.3 Asuncion

A location was visited 65km North of Asuncion (Ganadere Franco-Estancia, cattle ranch). This area was primarily used for cattle ranching and characterised by extensive grasslands and palm forest. The two dominant palm species were *Copernicia alba* (saline tolerant) and *Trithrinax schizophylla*. These palm forests

stretch for 300km into the southern Chaco. Much of this area becomes periodically flooded in winter months. In future text this location will be referred to as GF-ranch.

#### **4.3 Objectives**

1. To capture sylvatic triatomine bugs from the selected field sites, identify the species and provide them for morphometric analysis (Rojas de Arias and Patterson).
2. To isolate *T. cruzi* from sylvatic and domestic triatomine bugs and phenotype/genotype the isolates to subgroup level.
3. To test for multiclonality of *T. cruzi* infections in individual sylvatic and domestic triatomine bugs by direct plating of infected faeces and phenotyping/genotyping biological clones.
4. To capture sylvatic mammals especially edentates, isolate and phenotype/genotype *T. cruzi*.
5. To enhance understanding of the ecotopes and host associations of *T. cruzi* sublineages in Paraguay and of the actual or potential links between sylvatic and domestic transmission cycles.



# DEPARTAMENT OF BOQUERON

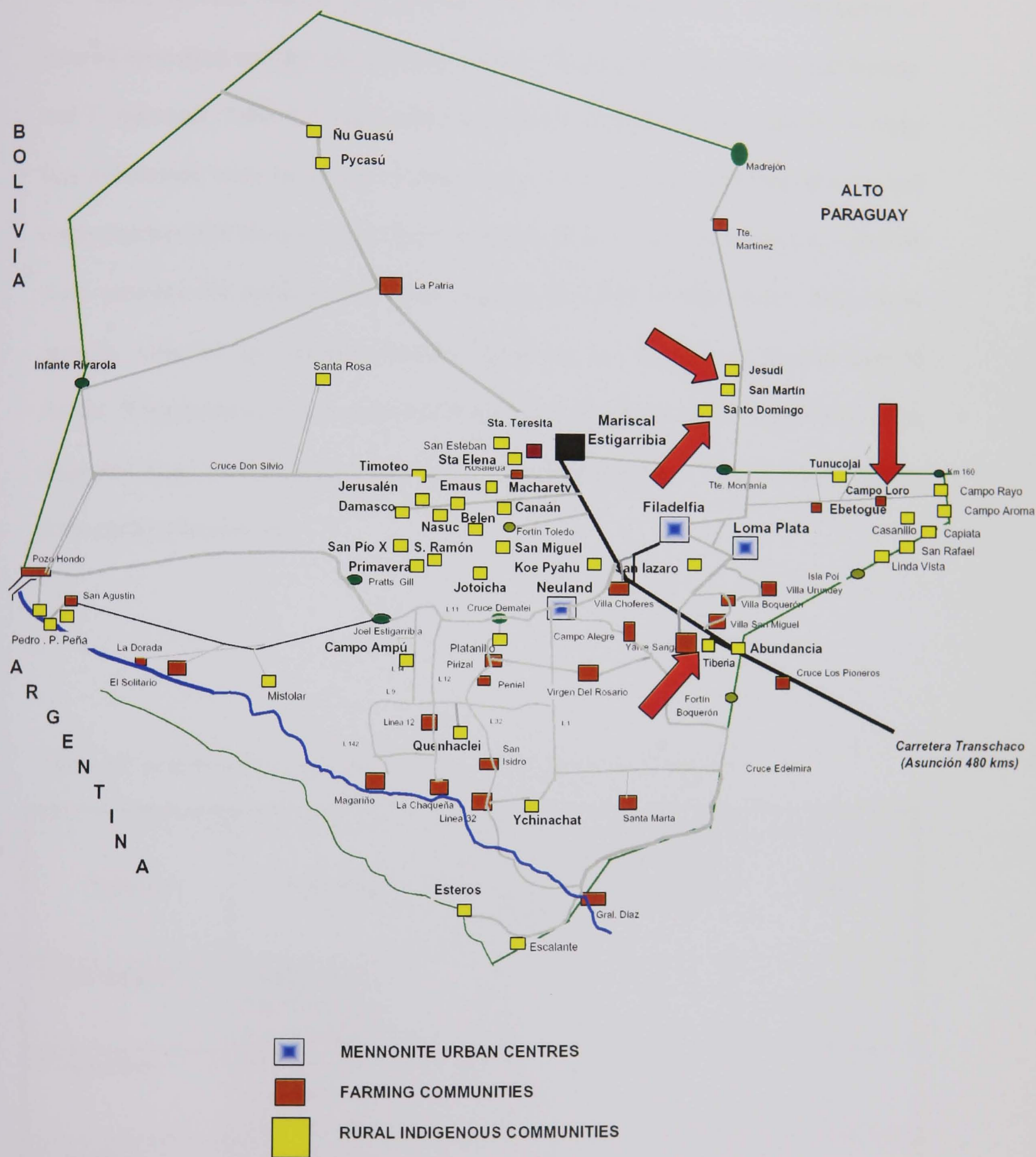


Figure 34 - Map of study sites in Boqueron in the Western Chaco. Red arrows show community locations.

## 4.4 Results

### 4.4.1 Collection of sylvatic triatomine bugs

Three sylvatic species of triatomine bug were found using a combination of manual dissection and the use of Noireau traps. These were *T. sordida*, *T. guasayana*, and *T. infestans*. Table 4.1 summarises the capture numbers. Study areas for sylvatic bug collections were Boqueron (Campo Lorro, San Domingo and San Martin) and cattle ranches (GF-Ranch) 65km from Asuncion. Due to time limitations no searches were possible for sylvatic triatomine bugs in the Dept of San Pedro. Bugs were initially screened for trypanosome-like organisms by microscopic examination of faeces. If trypanosomes were observed bugs were deemed positive. Inoculation from dissected bugs, to biphasic growth media, was performed for 10% of captured bugs from each collection site.

Table 4.1 A summary of sylvatic triatomine bugs found in Paraguay

Species	Locations	Sex/developmental stage			Total
		Adult		Nymphs	
		♂	♀		
<i>T. guasayana</i>	Campo Loro San Domingo San Martin	37	9	5	51
<i>T. infestans</i>	San Martin San Domingo Campo Loro	27	8	10	45
<i>T. sordida</i>	Campo Loro San Domingo San Martin GF-Ranch	24	3	0	27

#### **4.4.2 Collection of sylvatic *T. sordida***

Twenty seven adult, sylvatic *T. sordida* were collected from 4 locations (Campo Lorro, San Domingo, San Martin, and 3 ranches in the southern Chaco 65km North of Asuncion). Bugs were identified using morphometric keys (Lent & Wygodzinsky, 1979). No nymphs were detected. Ecotopes where bugs were captured were similar. Specimens were captured under the bark of fallen trees compacted with mud. After dissection of one site (Campo Lorro) a false coral snake, an unidentified species of frog, and rodent faeces were found. All bugs were dissected and none found positive for *T. cruzi*. At this site *T. guasayana* and *T. infestans* were also found sharing the same ecotope. At a single location (GF-Ranch), *T. sordida* was the only species found in the bark of a fallen palm tree (13 adults). This is the most southerly locality of our study area. Interestingly Noireau traps were placed in 80 standing palms (4 traps per tree) of two species (*C. alba* and *T. schizophylla*) all of which were negative for bugs. This suggests the ecotope of *T. sordida* is terrestrial rather than arboreal, or at least not palm associated. Samples from dissected bugs were preserved on filter paper for future blood meal analysis.

#### **4.4.3 Collection of sylvatic *T. guasayana***

Fifty one bugs were captured from the Chaco region in sylvatic areas surrounding the villages of San Martin, San Domingo and Campo Lorro. They were found in association with *T. sordida* in all areas studied in the Department of Boqueron. Furthermore, in areas surrounding San Domingo and San Martin they were also found in association with sylvatic *T. infestans*. Forty six specimens were adults (37 males, and 9 females) and 5 nymphs. All adults were dissected and found to be negative for *T. cruzi* by microscopy. Bugs were found in fallen trees and low lying



scrub which was compacted with soil. Faecal samples were preserved on filter paper for future blood meal analysis. One adult and one nymph of *T. guasayana* were found in an excavated uninhabited armadillo burrow.

#### **4.4.4 Collection of sylvatic *T. infestans***

Collection areas were 300 metres from the nearest peridomestic/domestic structure. Bugs were initially collected by locals asked to collect sylvatic specimens. Collection areas were revisited by our team and redissected to confirm the presence of sylvatic *T. infestans* (Figure 35). Proximal human and peridomestic dwellings had been sprayed within the previous twelve months (Rojas de Arias, personal communication).

A total of 45 sylvatic *T. infestans* were captured from two study locations (San Martin and San Domingo). These consisted of 35 adults (27 males and 8 females), and 10 nymphs. Bugs were identified using morphometric keys (Lent & Wygodzinsky, 1979). Five *T. infestans* were found to be positive for trypanosome like organisms by microscopic examination of faeces. Of these, 2 bug isolates were successfully grown *in vitro* for further characterisation. Interestingly for all 3 bug species captured, male adults were more common than females (ratios were 4:1 *T. guasayana*, 3.4:1 *T. infestans*, 8:1 *T. sordida*). Fewer nymphs than adult males were also obtained. However these were more difficult to capture due to their small size and local collectors were not aware that nymphs were the immature form of the adults. Therefore inferences based on the observed ratios of nymphs to adults cannot be relied upon.



Figure 35 - Sylvatic collection site (Campo Lorro) infested with *T. guasayana*, *T. infestans*, and *T. sordida*.

#### 4.4.5 Collection of domestic triatomines

Thirty two specimens of *T. sordida* were obtained from peridomestic chicken coops in 5 rural villages surrounding the town of San Pedro. Bugs were dissected, and examined microscopically for the presence of *T. cruzi* and nutrient slopes inoculated with faeces. All bugs were negative for *T. cruzi*. One hundred and fourteen households were also inspected for infestation by *T. infestans*, in rural dwellings surrounding San Pedro. One adult male *T. infestans* was found which was not infected with *T. cruzi*. These domestic premises had been sprayed within the previous twelve months. In a second field trip 29 infected specimens of domestic *T. infestans* were selected from captures from in 3 areas of Paraguay (see next section). Isolates from these bugs were used in domestic characterisation studies.

#### **4.4.6 Domestic isolates of *T. cruzi***

All samples were isolated from domestic *T. infestans* (from 4.4.5) with clonal colonies obtained by direct plating. A total of 29 bugs were used in further analysis with clonal colonies obtained from 16 bugs by direct plating. Overall 90 domestic isolates, inclusive of the biological clones were characterised. Table 4.8 shows origins of domestic isolates obtained from 3 regions of Paraguay (Figure 29). These were the Chaco region (17 bugs), the Department of San Pedro (2 bugs) and the Department of Paraguari (8 bugs). The origins of a further two bugs (Placa 1 and Placa 17) could not be sourced at the time of writing.

#### **4.4.7 Collection of sylvatic mammals**

A total of 64 mammals were captured in Paraguay. Four different armadillo species were obtained (*Chaetophractus* sp, *D. novemcinctus*, *E. sexinctus* and *T. matacus*), 2 marsupial species (*Monodelphis domestica*, *D. abiventris*) and 2 specimens of an unidentified bat species (Table 4.2).

Animals were initially tested for infection by a combination of thick films, xenodiagnosis, direct plating of whole and centrifuged blood, or by inoculation of whole blood into biphasic growth medium. Animals were deemed positive if any of the aforementioned techniques revealed the presence of trypanosomes. Table 4.3 shows animals, at an individual level, positive for *T. cruzi*.



Table 4.2 Mammal species captured in Paraguay

Mammal species	Number captured	Positive for <i>T. cruzi</i>
<i>Dasypus novemcinctus</i>	21	13
<i>Euphractus sexinctus</i>	13	2
<i>Didelphis albiventris</i>	3	0
<i>Tolypeutes matacus</i>	14	0
<i>Monodelphis domestica</i>	3	1
<i>Chaetophractus</i> spp	8	0
Bat (unidentified)	2	0

#### 4.4.8 *T. cruzi* isolates from sylvatic mammals

61.9% of *D. novemcinctus* specimens were positive for *T. cruzi*, 15.4% of *E. sexinctus* and 33% of *M. domestica*, although the sample size consisted of only three animals in the latter case. Interestingly megatrypanosomes were also observed in blood films of 2 of 3 *M. domestica*, but direct culture methods failed to produce *in vitro* growth. Specimens of *T. matacus* were negative for *T. cruzi* as were 8 specimens of *Chaetophractus* spp. It should be noted that these are conservative infection estimates. Some animals were not tested by xenodiagnosis due to lack of colony reared *T. infestans* (3 of 21 *D. novemcinctus*; 3 of 11 *T. matacus*; 7 of 8 *Chaetophractus* spp; 3 of 13 *E. sexinctus*, and 1 of 3 *D. albiventris*). In these cases direct inoculation into biphasic growth media was performed. However xenodiagnosis followed by bug dissection was the most sensitive method of detecting positive mammals. With one exception (ARMA 20) all positive isolates were obtained by xenodiagnosis. Direct plating methods using whole and centrifuged blood failed to produce growth when applied to mammals (13 animals tested, 6 positive by

xenodiagnosis). Only 5 of 16 (31%) positive animals were detected by the examination of blood films or by centrifugation of whole blood in haematocrit tubes. Table 4.3 shows the collection locations of those sylvatic isolates successfully expanded *in vitro*.

#### 4.5 Characterisation of *T. cruzi* isolates

Domestic and sylvatic isolates were characterised by a combination of mini-exon and large and small subunit rRNA PCR profiling and enzyme electrophoresis.

Table. 4.3 Sylvatic field isolates of *T. cruzi* used in the study

Isolate	Method of isolation	Host	Origin
SP4	Xenodiagnosis	<i>Monodelphis domestica</i>	Dept of San Pedro
SP13	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
SP14	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
SP15	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
SP16	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
ARMA 134	Xenodiagnosis	<i>Euphractus sexinctus</i>	Dept of San Pedro
ARMA 34	Xenodiagnosis	<i>Euphractus sexinctus</i>	Dept of San Pedro
ARMA 9	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco
ARMA 12	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco
ARMA 13	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco
ARMA 18	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco
ARMA 24	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
ARMA 25	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
ARMA 26	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
ARMA 27	Xenodiagnosis	<i>Dasypus novemcinctus</i>	Dept of San Pedro
Pot7a	Direct inoculation	Sylvatic <i>T. infestans</i>	San Martin, Chaco
Pot7b	Direct inoculation	Sylvatic <i>T. infestans</i>	San Martin, Chaco

A further specimen (ARMA 20, *D. novemcinctus* from Campo Lorro) was positive for *T. cruzi* by direct culture. This culture was maintained in Paraguay and unavailable at the time of characterisation.



#### 4.5.1 Characterisation of Paraguayan stocks by large and small subunit rRNA and mini-exon genes

A total of 90 parental and clonal isolates from 31 bugs and 15 animals were characterised by mini-exon and large and small subunit rRNA PCR profiling (Methods 2.10). Reference strains representing each of the 6 previously defined lineages were used (Brisse *et al.*, 1999). These were X10 cl1 (TC1), CAN III cl1 (TC2a), Esm cl3 (TC2b), X109/2 and X9/3 (TC2c), 92.80 cl1 (TC2d) and CL Brener (TC2e). Profiles of PCR products obtained for each of these reference strains are shown in Figure 36.

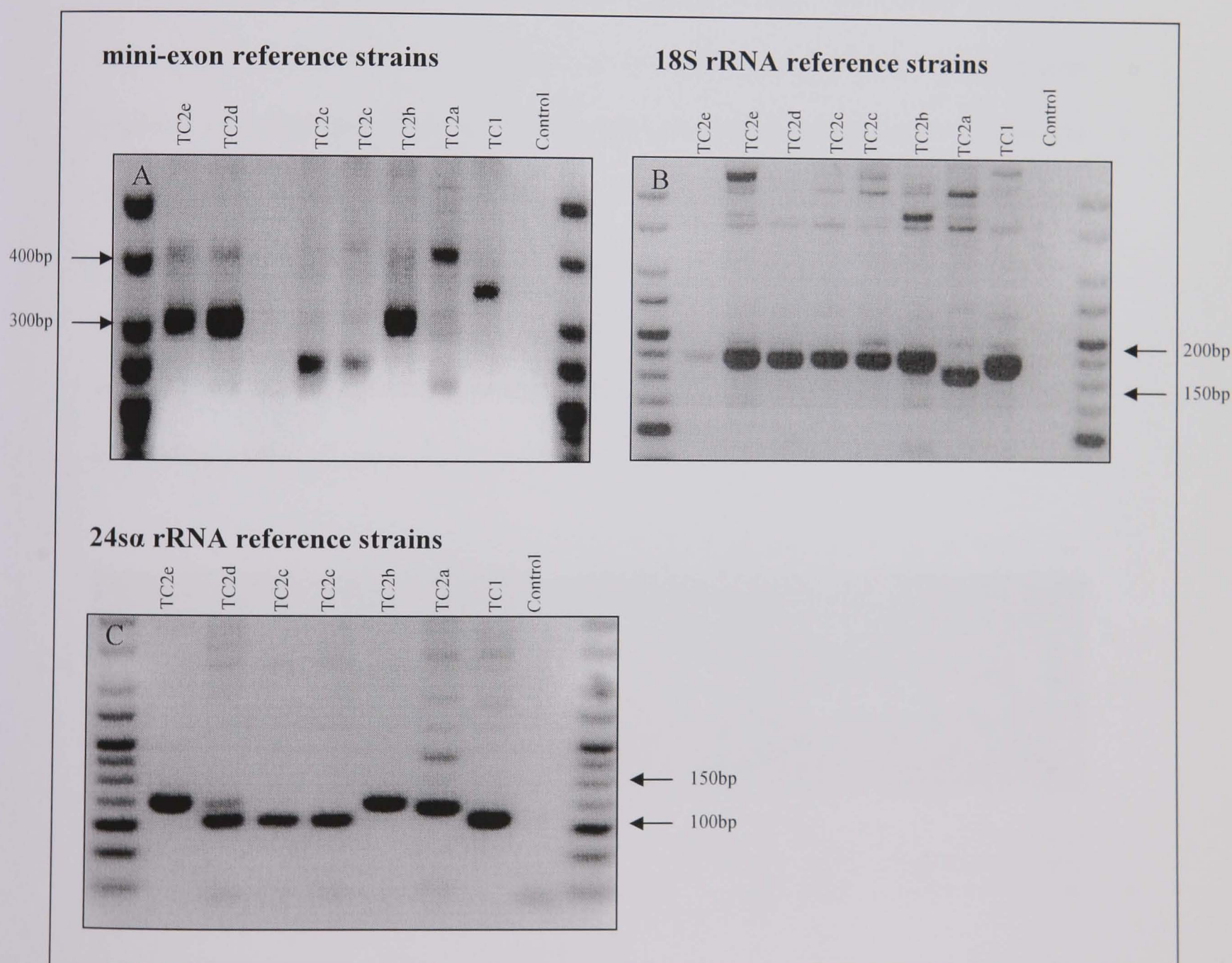


Figure 36 - Reference strain profiles, A. mini-exon, B. 18S rRNA and C. 24S $\alpha$  rRNA characterisation. Origin is at the top of the picture in each case. Two reference isolates (TC2e) were used at different concentrations in the 18S rRNA to check primer stocks. The lighter band denotes amplification products using older primers. These primers were subsequently discarded.



4.5.2 PCR amplification of the non-transcribed spacer region of the mini-exon gene

The following mini-exon PCR products were produced for each representative reference strain: TC2c (250bp with no or weak amplification at 300bp), TC2b, d, e (300bp), TC1 (350bp) with TC2a producing a characteristic 400bp band with a lack of or weak amplification product at 300bp (Figure 36A, 37). Characterisation using the markers in the region of 250bp to 400bp was robust and reproducible.

Table 4.4 summarises the reaction products produced during amplification. All isolates (parental and clonal) from domestic triatomine bugs, two sylvatic triatomine bugs and 3 mammal isolates (ARMA 25,34, and 134) produced amplification products of 300bp and could be initially characterised as belonging to sublineages TC2 b, d or e. Figure 33 shows an example of one such profile. Twelve of 15 sylvatic mammal isolates did not produce an amplification product at 300bp. The lack of amplification at 300bp suggests they belong to lineage TC2c. This is further evidenced by the correspondence with TC2c reference strain profiles and the presence of a 250bp product (Figure 36A and 38).

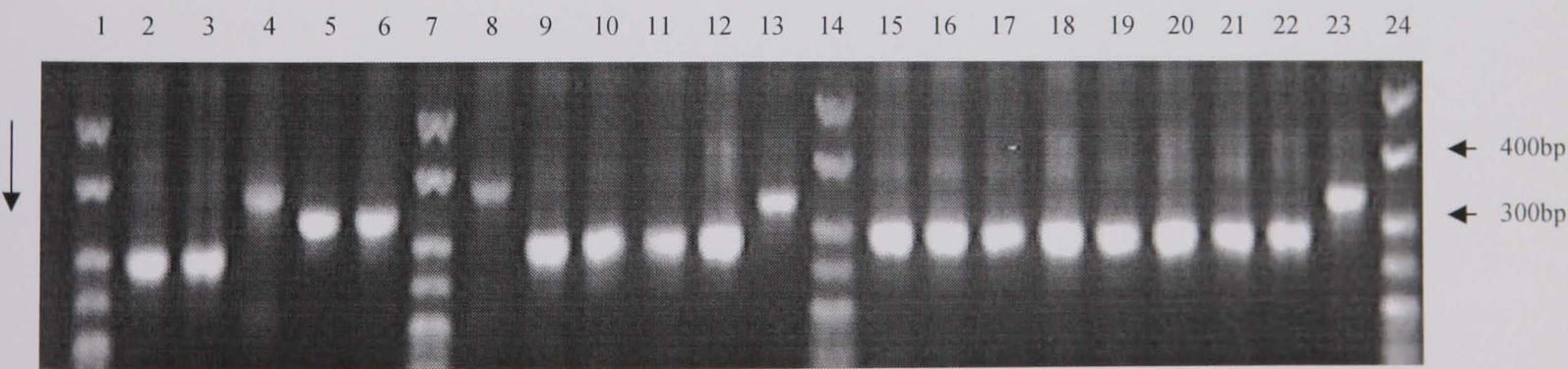


Figure 37 - Mini-exon PCR amplification products of domestic isolates from Paraguay. TC1 reference strain produced a 350bp product (lanes 5,6,13 and 23). TC2b reference strain produced bands of 300bp (lanes 2 and 3). TC2e reference strain produced a single 400bp product (lanes 4 and 8). All domestic isolates produced a 300bp product (lanes 1-2, 9-12 and 15-22).





Figure 38 - Mini-exon PCR amplification products. Lanes 2 and 11 contain TC1 reference strains; Lanes 3 and 4 denote TC2b and TC2d reference strains; lane 5 contains reference strain TC2c. Lanes 6-9 contain sylvatic mammal isolates (ARMA 9,18,24 and 27). Lane 10 contains a 300bp product profile produced from ARMA 25. Origin is at the top of the figure. Control is in lane 13. Origin is at the top in each case.

#### 4.5.3 PCR amplification of the size-variable domain of the 18S rRNA sequence

Characterisation of the 18S rRNA resulted in profiles distinguishing reference strains TC2a (155bp), with TC2b, TC2c, TC2d and TC2e producing a 165 bp product (Table 4.4). All domestic and sylvatic samples produced amplification products of 165bp suggesting all isolates belong to substrains TC2b, c, d or e using this marker. Figure 39 shows an example of a profile containing domestic and sylvatic isolates. Interestingly, and in contrast with Brisse *et al.* (2001) no distinction could be made between TC2b and TC2e using 18S rRNA PCR (Figure 40). They reported a lack of amplification of TC2e in the 165bp region. This was the least useful marker for resolving strains.

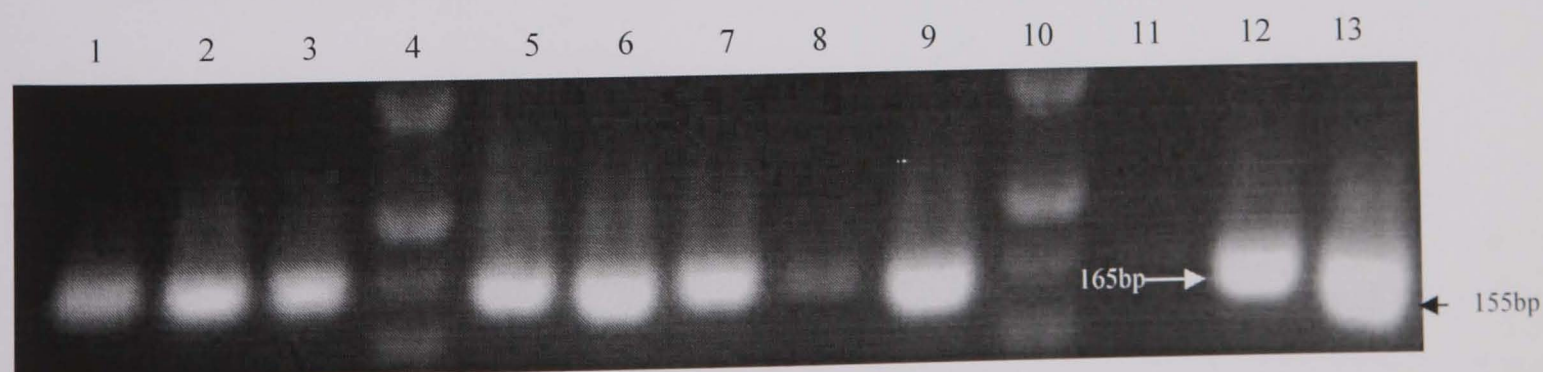


Figure 39 -18S rRNA PCR profile of domestic and sylvatic isolates. Order of loading from left to right . Lanes 1-3 and 5-9 show identical 165bp amplification products typical of domestic and sylvatic isolates in this study. Sample in lane 11 did not amplify. Lane 12 contains TC2e reference strain and lane 13 contains the TC2a reference strain showing a 155bp product.



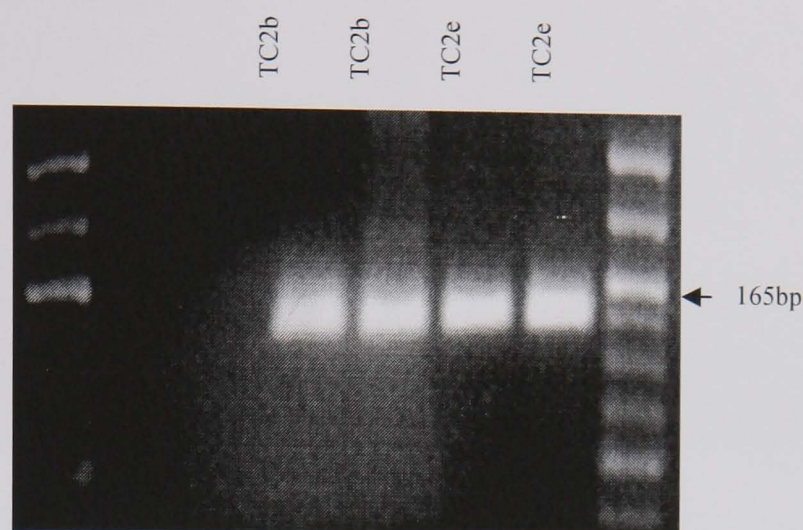


Figure 40 - PCR amplification of the size-variable domain of the 18S rRNA sequence. Strains TC2b and TC2e both show 165bp amplification products in contrast to Brisse *et al.*, (2001).

Table 4.4 Amplification product profiles (bp) and strain resolution for mini-exon, and large and small subunit rRNA PCR

	TC1	TC2a	TC2b	TC2c	TC2d	TC2e	Subgroup resolution (TC2)
<b>Mini-exon</b>	350bp	400bp <sup>a</sup>	300bp	250bp <sup>b</sup>	300bp	300bp	TC2a and c
<b>18S rRNA</b>	175bp	155bp	165bp	165bp	165bp	165bp	TC2a
<b>24S rRNA</b>	110bp	120bp	125bp	110bp	110/125bp	125bp	TC2a,c,d

<sup>a</sup> With no amplification at 300 or 350bp regions. <sup>b</sup> With no amplification at 300bp

#### 4.5.4 PCR amplification of the D7 divergent domain of the 24S $\alpha$ rRNA

24S $\alpha$  rRNA amplification products differentiated between four lineages; TC1 and TC2c (110bp), TC2d (110bp with an extra band at 125bp), TC2b and TC2e (125bp) and TC2a (120bp). Table 4.4 summarises these results. Figure 41 shows a profile of selected samples from both sylvatic and domestic sources.



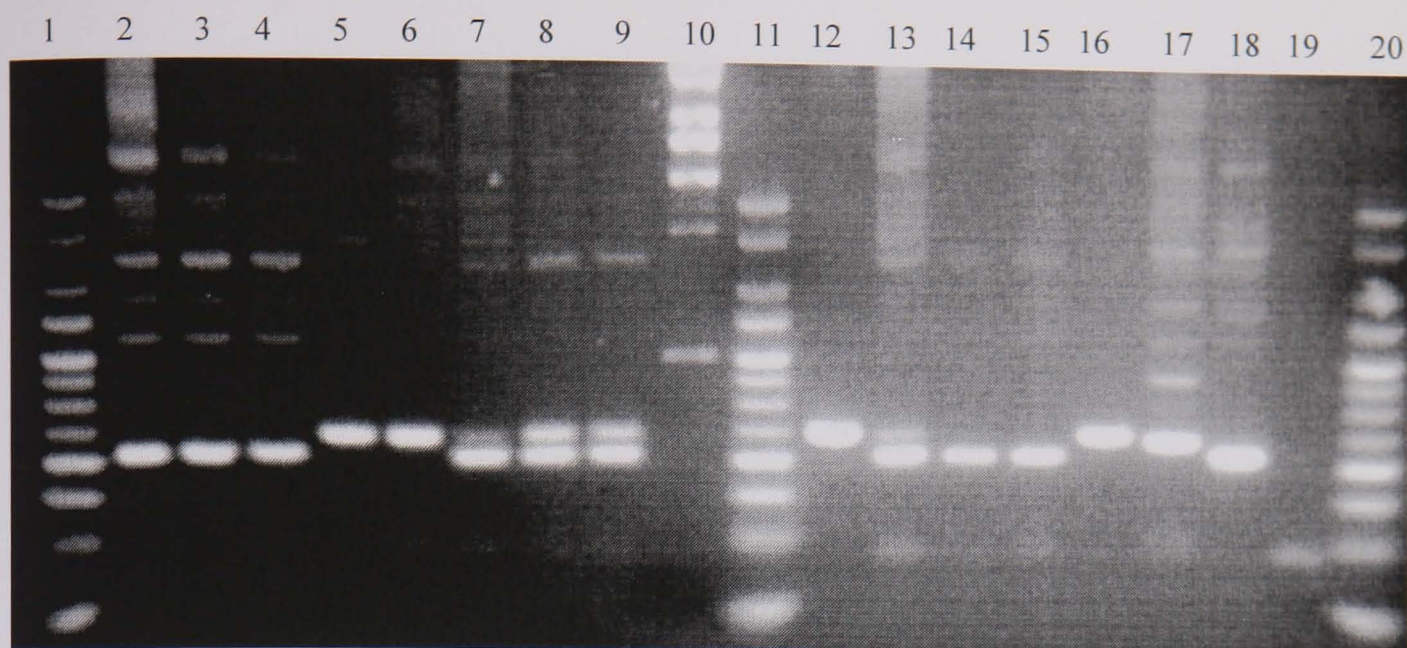


Figure 41 - Amplification of the 24S $\alpha$  rRNA divergent domain showing Paraguayan isolates in comparison to reference strains. Order of loading from left to right is lane 1, 0.5kb ladder; lane 2, SP14; lane 3, SP13; lane 4, ARMA 27; lane 5 TC2b; lane 6, ARMA 34; lane 7, Chaco 2; lane 8, Para 3; lane 9, ARMA 25; lane 10, 1kb ladder; lane 11 0.5kb ladder; lane 12, TC2e; lane 13, TC2d; lane 14, TC2c; lane 15, TC2c; lane 16, TC2b; lane 17, TC2a; lane 18 TC1; lane 19 Control; lane 20, 0.5kb ladder.

Profiling using 24S $\alpha$  rRNA PCR was the most informative marker, revealing that domestic isolates Para1-Para7, Chaco2, ARMA 134 and ARMA 25 belong to lineage TC2d. Additionally these results were in agreement with mini-exon profiles with regard to the remaining ARMA samples categorised as TC2c. No evidence was found of the presence of TC2a. All other domestic isolates, a single sylvatic mammal isolate (ARMA 34) and the two sylvatic isolates from sylvatic *T. infestans* were either TC2b or TC2e.

#### 4.5.5 Summary

In summary, by using a combination of mini-exon, and large and small subunit rRNA amplification reactions four of the subspecific groups could be resolved (Figure 42). These were TC1, TC2a, TC2c, TC2d. TC2b and e could not be resolved using these amplification markers. Confirmatory makers are shown in Table 4.4. Thus 83 domestic isolates and two isolates from sylvatic *T. infestans* could be characterised as belonging to TC2b or TC2e along with one sylvatic isolate from *E. sexinctus* (ARMA 34). Eight domestic isolates Para1-7 and Chaco 2 and two sylvatic armadillo isolates



(*D. novemcinctus*, ARMA 25; *E. sexinctus*, ARMA 134) show characteristics of sublineage TC2d. Twelve of the 15 mammal isolates corresponded with lineage TC2c reference strain. All but one of twelve isolates from *D. novemcinctus* belonged to lineage TC2c and the remaining isolate to Tc2b or TC2e. Tables 4.5 and 4.7 show isolate subgroup results after further characterisation by isoenzyme analysis.

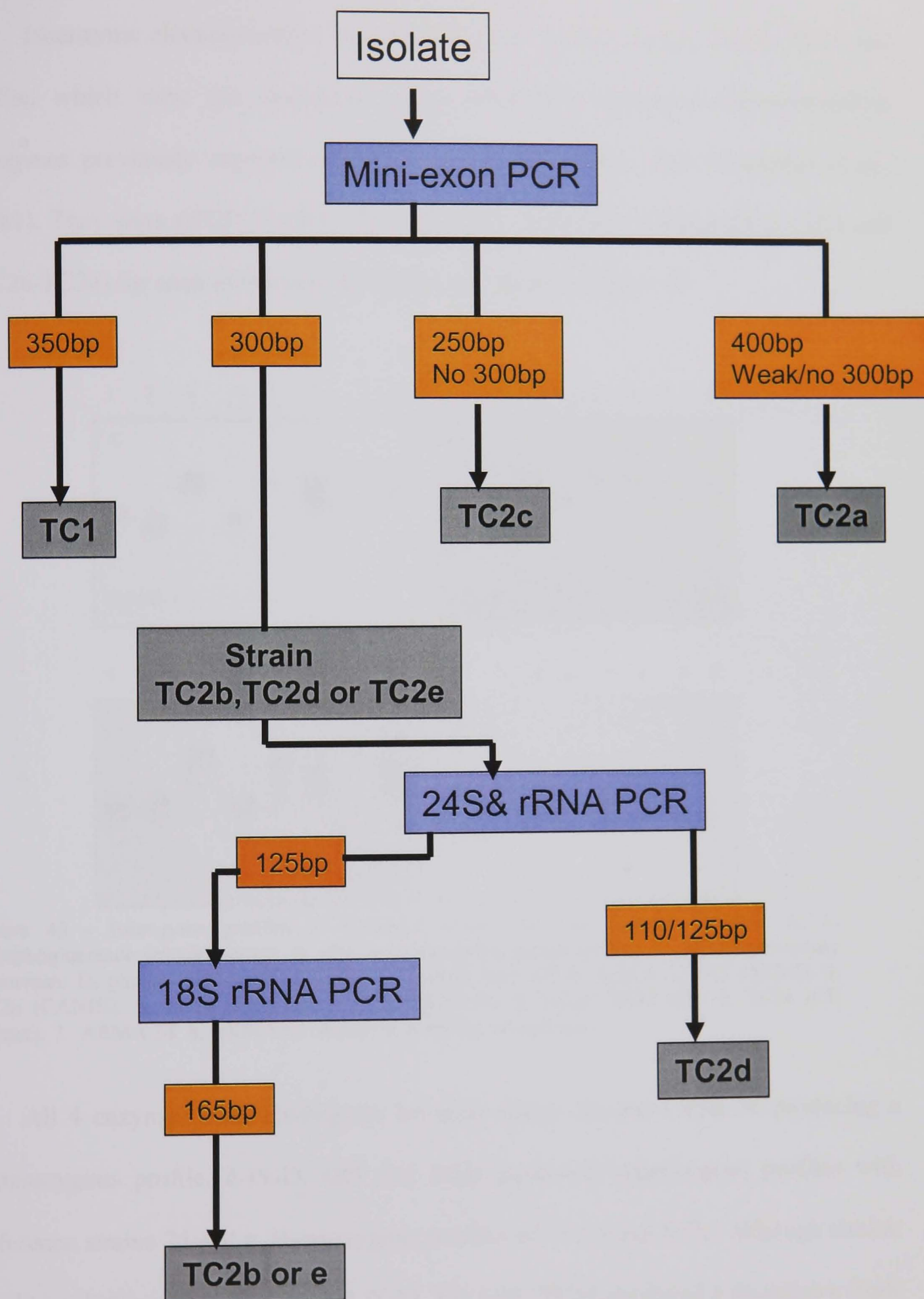


Figure 42 - A flow chart to demonstrate the ability to resolve 4 of 6 groups of *T. cruzi* by mini-exon and large and small subunit rRNA. See table 4.4 for confirmatory markers.

#### 4.6 Isoenzyme characterisation of Paraguayan isolates

Isoenzyme characterisation was undertaken to resolve sample strains TC2b and TC2e, which were not elucidated using rRNA and mini-exon characterisation. Enzymes previously reported to resolve these strains were used (Chapman *et al.*, 1981). They were 6PGD, G6PD, GPI, and PGM. Reference strain profiles (TC1 and TC2a-TC2e) for each of the enzymes tested are shown in Figure 43.

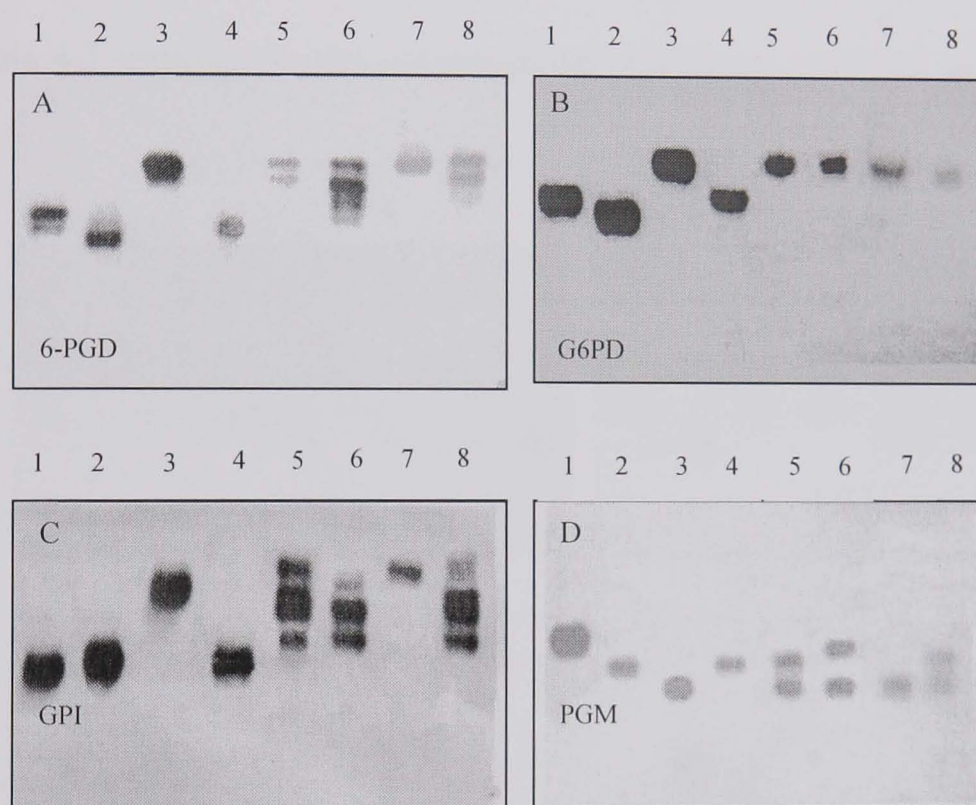


Figure 43 - Isoenzyme profiles of reference strains for four enzyme systems. A, 6-phosphogluconate dehydrogenase. B, glucose-6-phosphate dehydrogenase. C, glucosephosphate isomerase; D, phosphoglucomutase. Order of loading from left to right is 1, TC1 (X10/1). 2, TC2a (CANIII). 3, TC2b (Esmcl2). 4, TC2c (X109/2). 5, TC2d (92.80 cl1). 6, TC2e (CL Brener). 7, ARMA 34. 8, ARMA 25. Anode is at the top in each case.

All 4 enzymes could distinguish between strains 2b and e with 2e producing a heterozygous profile. 6-PGD, GPI and PGM produced heterozygous profiles with reference strains 2d and e. Heterozygous profiles of TC2d and TC2e, although similar could be distinguished by GPI and PGM. For GPI, TC2d produced a distinctive triple banded profile when compared to the triple banded profile from TC2e: TC2d produced a heterozygous profile with the two bands separated by a greater electrophoretic distance than TC2e, and for PGM the reverse was true (Figure 43).



#### 4.6.1 Characterisation of *T. cruzi* isolates from mammals

SP4, 13, 14, 15, 16 and ARMA 9, 12, 13, 18, 24, 26, 27 produced profiles resembling TC2c (in agreement with mini-exon profiles) with all four enzymes (Figure 44). Isolates ARMA 25 and ARMA 134 were related to TC2d, in agreement with mini-exon and large and small subunit rRNA analysis by all four enzyme systems (Figure 43). Additionally ARMA 34 produced a profile resembling that of TC2b. Table 4.5 summarises the subgroups obtained from mammal isolates.

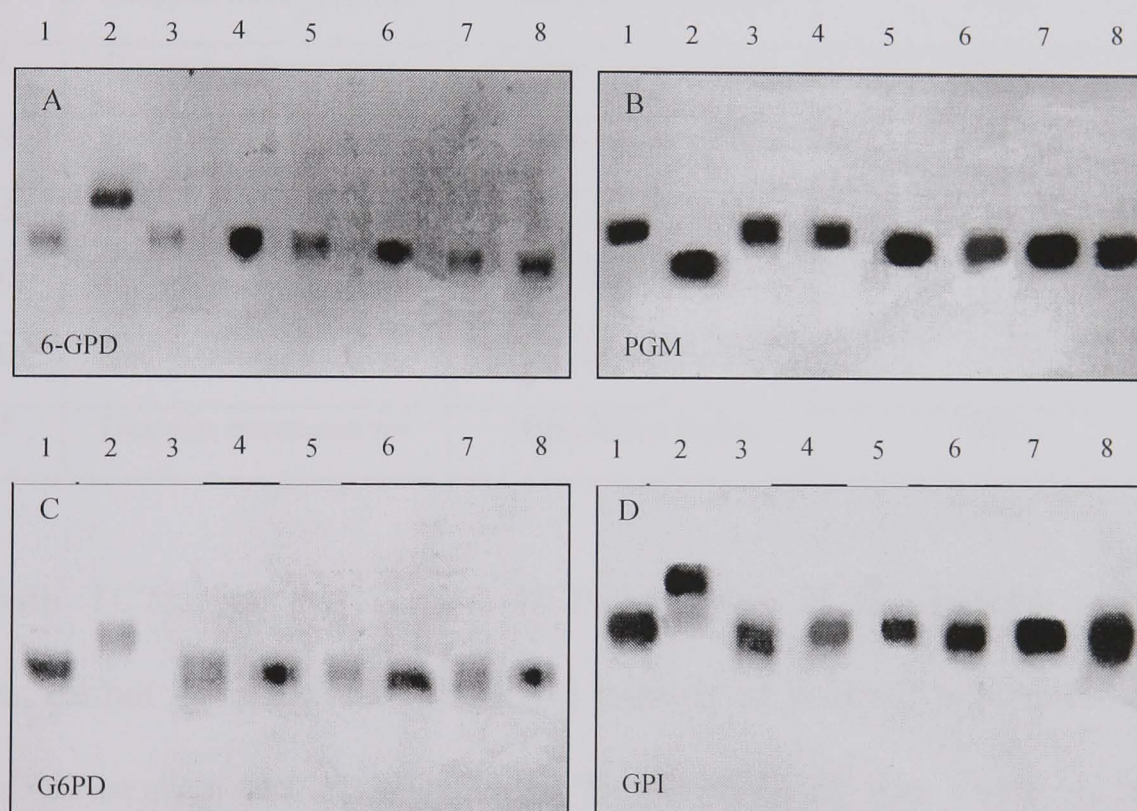


Figure 44 - Isoenzyme profiles of *T. cruzi* from sylvatic mammal isolates. A, 6-phosphogluconate dehydrogenase. B, phosphoglucomutase. C, glucose-6-phosphate dehydrogenase. D, glucosephosphate isomerase. Samples from left to right are: 1, X109/2 (TC2c reference). 2, Esm cl2 (TC2b reference). 3, SP4. 4, SP13. 5, ARMA 9. 6, ARMA 13. 7, ARMA 26. 8, ARMA 24.

Thus *D. novemcinctus* was found infected with *T. cruzi* in both San Pedro and the Chaco region; eleven were infected with TC2c and single specimen with TC2d (San Pedro). One specimen of the marsupial *M. domestica* was also infected with TC2c from the Department of San Pedro. In contrast *E. sexinctus* was not found



Table 4.5 Strains of *T. cruzi* obtained from sylvatic mammals

Isolate	Host	Origin	Strain
SP4	<i>Monodelphis domestica</i>	Dept of San Pedro	TC2c
SP13	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2c
SP14	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2c
SP15	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2c
SP16	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2c
ARMA 134	<i>Euphractus sexcinctus</i>	Dept of San Pedro	TC2d
ARMA 34	<i>Euphractus sexcinctus</i>	Dept of San Pedro	TC2b
ARMA 9	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco	TC2c
ARMA 12	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco	TC2c
ARMA 13	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco	TC2c
ARMA 18	<i>Dasypus novemcinctus</i>	Campo Lorro, Chaco	TC2c
ARMA 24	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2c
ARMA 25	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2d
ARMA 26	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2c
ARMA 27	<i>Dasypus novemcinctus</i>	Dept of San Pedro	TC2c

infected with TC2c, but one carried TC2b and one TC2d. Specific host/strain associations cannot be made with the small number of infected mammals of some species (*M. domestica* and *E. sexcinctus*). However TC2d was found in both *D. novemcinctus* and *E. sexcinctus*. Additionally strain TC2c was identified in *D. novemcinctus* and *M. domestica*. It appears that the most prevalent strain of *T. cruzi* infecting *D. novemcinctus* is TC2c over a wide geographical area in the sylvatic cycle.

#### 4.6.2 Characterisation of domestic isolates

Isolates were initially screened using the enzyme GPI, which showed an obvious profile difference between TC2b and TC2e, TC2e producing a heterozygous profile (Figure 43C). Samples were tested from 29 bugs; biological clones were obtained from 16 of these bugs (Table 4.6). Inclusive of biological clones a total of 90 isolates were tested.

Parental isolates from 29 bugs were initially examined. Parental isolates are those obtained prior to plating by inoculation of bug faeces on to growth media (Table 4.7). Results revealed a high prevalence of TC2e. Isolates from 16 of 29 bugs (55.2%) demonstrated a heterozygous profile consistent with reference strain TC2e, from both Chaco and San Pedro collection sites. This suggests a wide geographic spread of this genotype. TC2b profiles were present in 5 bugs (17.2%) from the Chaco region and San Pedro. Strain TC2d (previously resolved by mini-exon, large and small subunit rRNA) was found in 8 bugs (25.6%) collected from the Chaco and Paraguari. Figure 45 shows examples of GPI profiles of domestic samples.

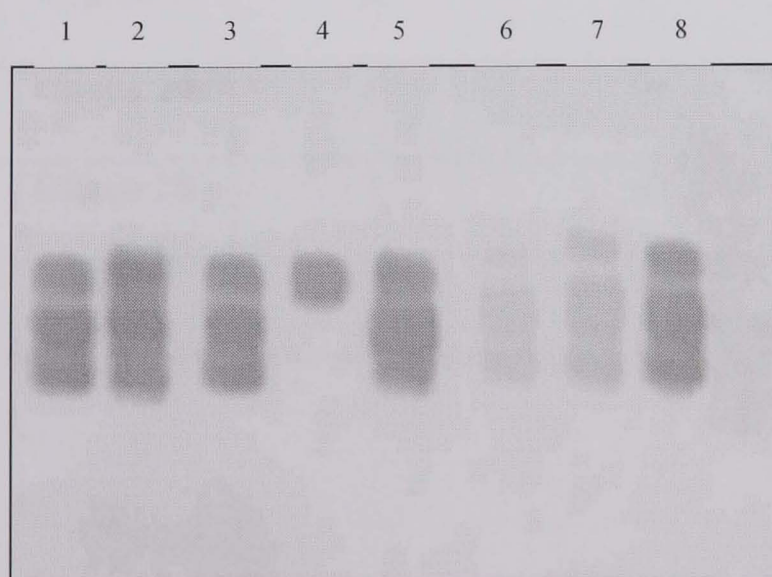


Figure 45 - Isoenzyme profile of domestic TC2e, TC2b and TC2d isolates using the glucosephosphate isomerase enzyme system. Order of loading from left to right is 1, Chaco15 (TC2e). 2, Chaco17 (TC2e). 3, Chaco21 col 1 (TC2e). 4, Chaco 25 col 1 (TC2b). 5, Chaco 14 (TC2e). 6, Chaco 21 col 1. 7, Para2 (TC2d). 8, Chaco2 (TC2d). Origin is at the top in each case.



### 4.6.3 Characterisation of clonal colonies from domestic isolates

Four of the 16 bugs from which clonal colonies were obtained appeared to harbour mixed infections (TC2b with TC2d) shown in table 4.6. Three were *T. infestans* collected from the Chaco region (Chaco 9, 23 and 28) and one *T. infestans* from San Pedro (SPE3). Figure 46 shows a summary GPI profile. Confirmatory enzyme profiling was undertaken on these samples using PGM and 6-PGD systems (Figure 47). Interestingly, none of the parental isolates (inoculated into growth media immediately prior to plating) demonstrated mixed profiles of TC2e and TC2b. However this is not surprising as TC2b profiles would be at least partially obscured by TC2e profiles with the enzymes used. All four parental isolates had profiles resembling TC2e.

Table 4.6 Domestic *T. infestans* harbouring mixed infections from a total of 29 bugs examined.

Bug number	Bug isolate	No colonies characterised as Tc2b	No colonies characterised as Tc2e	Number of clonal colonies tested
1	Chaco 9	3	3	6
2	Chaco 23	1	7	8
3	Chaco 28	2	4	6
4	SPE3	1	2	3

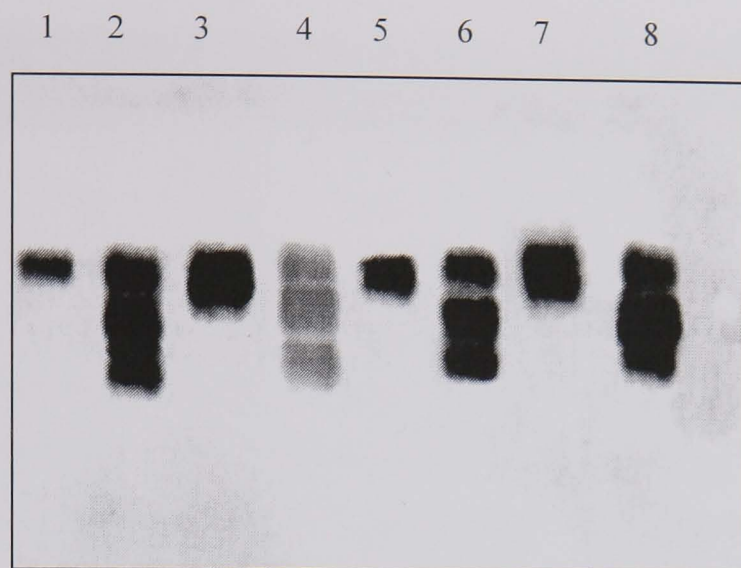


Figure 46 - Isoenzyme profile using the GPI enzyme system demonstrating multiclinality in single triatomine bugs. Order of loading from left to right is: lane 1, Chaco 9 col 2; lane 2, Chaco 9 col 14; lane 3, Chaco 23 col 4; lane 4, Chaco 23 col 6; lane 5 Chaco 28 col 3; lane 6, Chaco 28 col 9; lane 7, SPE3 col 9; lane 8, SPE col 10. Origin is at the top in each case.

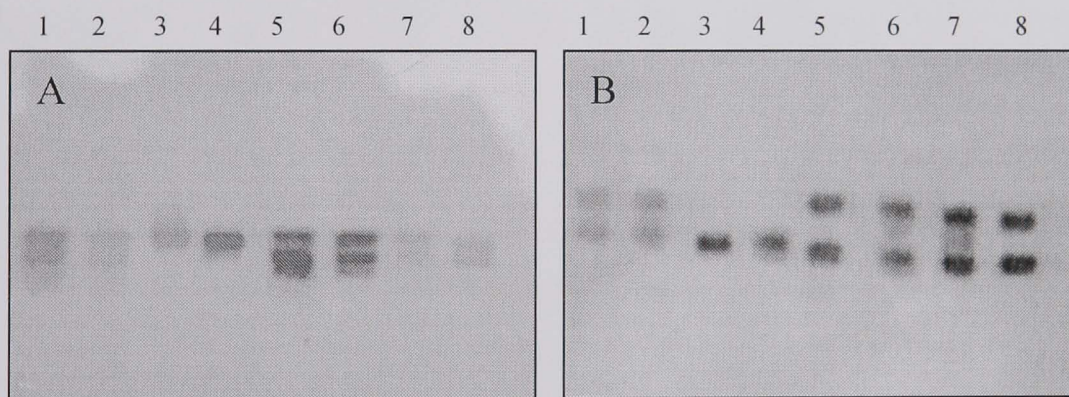


Figure - 47 Isoenzyme profiles of selected domestic isolates showing multiclinality. A, 6-phosphogluconate dehydrogenase. B, phosphoglucomutase. Order of loading from left to right is: lane 1, Chaco 2; lane 2, Para2; lane 3, SPE3 clone 9; lane 4, Chaco 23 clone 4; lane 5, SPE clone 10; lane 6, Chaco 23 clone 6; lane 7, Chaco 21; lane 8, SPE 1. Origin is at the top in each case.



Table 4.7 Strain classification of domestic and sylvatic triatomine bug isolates.

Sample No.	Sample name	Strain type	Sample No.	Sample name	Strain type	Sample No.	Sample name	Strain type	Sample No.	Sample name	Strain type
1	Chaco 1	TC2e	24	Chaco 17 col 8	TC2e	47	Chaco 24 col 1	TC2e	70	Chaco 31 col 5	TC2e
2	Chaco 2	TC2d	25	Chaco 17 col 10	TC2e	48	Chaco 24 col 2	TC2e	71	Chaco 31 col 6	TC2e
3	Chaco 3	TC2e	26	Chaco 20	TC2e	49	Chaco 24 col 3	TC2e	72	Chaco 31 col 7	TC2e
4	Chaco 4 col 8	TC2b	27	Chaco 21 col 1	TC2e	50	Chaco 24 col 4	TC2e	73	Chaco 31 col 13	TC2e
5	Chaco 9	TC2e	28	Chaco 21 col 2	TC2e	51	Chaco 24 col 5	TC2e	74	Chaco 31 col 14	TC2e
6	Chaco 9 col 2	TC2b	29	Chaco 21 col 3	TC2e	52	Chaco 24 col 10	TC2e	75	Para 1	TC2d
7	Chaco 9 col 3	TC2b	30	Chaco 21 col 4	TC2e	53	Chaco 24 col ?	TC2e	76	Para 2	TC2d
8	Chaco 9 col 5	TC2e	31	Chaco 21 col 5	TC2e	54	Chaco 25 col 1	TC2b	77	Para 3	TC2d
9	Chaco 9 col 7	TC2e	32	Chaco 21 col 6	TC2e	55	Chaco 25 col 6	TC2b	78	Para 3a	TC2e
10	Chaco 9 col 14	TC2e	33	Chaco 21 col 7	TC2e	56	Chaco 28	TC2e	79	Para 4	TC2d
11	Chaco 9 col 16	TC2b	34	Chaco 21 col 8	TC2e	57	Chaco 28 col 2	TC2e	80	Para 5	TC2d
12	Chaco 13	TC2b	35	Chaco 21 col 9	TC2e	58	Chaco 28 col 3	TC2b	81	Para 6	TC2d
13	Chaco 14	TC2e	36	Chaco 21 col 10	TC2e	59	Chaco 28 col 4	TC2b	82	Para 7	TC2d
14	Chaco 15	TC2e	37	Chaco 23	TC2e	60	Chaco 28 col 6	TC2e	83	Placa 1 col 1	TC2e
15	Chaco 15 col 4	TC2e	38	Chaco 23 col 2	TC2e	61	Chaco 28 col 7	TC2e	84	Placa 1 col 9	TC2e
16	Chaco 17	TC2e	38	Chaco 23 col 4	TC2b	62	Chaco 28 col 9	TC2e	85	Placa 17 col 4	TC2e
17	Chaco 17 col 1	TC2e	40	Chaco 23 col 6	TC2e	63	Chaco 30 col 2	TC2b	86	Placa 17 col 10	TC2e
18	Chaco 17 col 2	TC2e	41	Chaco 23 col 7	TC2e	64	Chaco 30 col 3	TC2b	87	SPE 1	TC2e
19	Chaco 17 col 3	TC2e	42	Chaco 23 col 8	TC2e	65	Chaco 30 col 7	TC2b	88	SPE 3 col 1	TC2e
20	Chaco 17 col 4	TC2e	43	Chaco 23 col 9	TC2e	66	Chaco 30 col 8*	TC2b	89	SPE 3 col 9	TC2b
21	Chaco 17 col 5	TC2e	44	Chaco 23 col 10	TC2e	67	Chaco 30 col 9	TC2b	90	SPE 3 col 10	TC2e
22	Chaco 17 col 6	TC2e	45	Chaco 23 col 11	TC2e	68	Chaco 30 col 11	TC2b	91	Pot7a	TC2b
23	Placa 17 col 7	TC2e	46	Chaco 24	TC2e	69	Chaco 31 col 2	TC2e	92	Pot7b	TC2b

Isolates were derived from *T. infestans*. Chaco, indicates domestic isolates from the Chaco region. Para, indicates domestic isolates obtained from the Department of Paraguari. SPE, indicate domestic isolates obtained from the Department of San Pedro. Placa, indicates isolates awaiting regional placement from collaborators in Paraguay. Col, represents clonal colonies isolated from bug faeces of individual triatomine bugs. Grey table cells ( ) represent parental isolates. Orange cells ( ) were isolates from sylvatic *T. infestans*. Strains in red typeface indicate mixed infections in single bugs.

#### 4.6.4 Summary of distribution of characterised isolates

Domestic and sylvatic isolates were characterised from two regions (Chaco and San Pedro), domestic samples were also characterised from Paraguari (Figure 48).

In this work San Pedro revealed a particularly diverse subset of strains. In the sylvatic cycle TC2c was isolated from *M. domestica*, and *D. novemcinctus*. TC2d from *E. sexcinctus* and *D. novemcinctus* and TC2b from a single specimen of *E. sexcinctus*. Domestic strains isolated were TC2b and TC2e. In the Chaco region sylvatic isolates were TC2c from *D. novemcinctus* (4 animals) and TC2b from 2 sylvatic specimens of *T. infestans*. In the domestic cycle TC2b, TC2d and TC2e were identified. Additionally, in the department of Paraguari 7 domestic isolates were characterised as TC2d.

Subgroup TC2e was the primary strain in domestic cycles (55.2% of bug isolates) in Chaco and San Pedro. In Paraguari TC2d was the only domestic strain isolated. Strains TC2b, c and d were isolated from sylvatic sources. TC2c appears to be the predominant but not only strain present in *D. novemcinctus* (61.9% of animals infected). *E. sexcinctus* (15.4% of animals infected) were infected with TC2b and TC2d were identified. TC2c was not identified in this species, however the low number of infected animals (2 of 13) does not discount the presence of more strains being identified in future studies. Therefore it appears these *D. novemcinctus* and *E. sexinctus* have significant roles to play as reservoirs of *T. cruzi* in sylvatic cycles in both the Chaco region and the Dept of San Pedro. *T. matacus* does not appear to be a significant reservoir of *T. cruzi*. Similarly no *Chaetophractus* specimens were positive although the fact that xenodiagnosis could not be performed on these animals does not exclude the possibility of future associations.



No positive isolates were obtained from sylvatic *T. guasayana* or *T. sordida*. Five specimens of sylvatic *T. infestans* were identified from a single location in the Chaco, two of which were infected with TC2b.

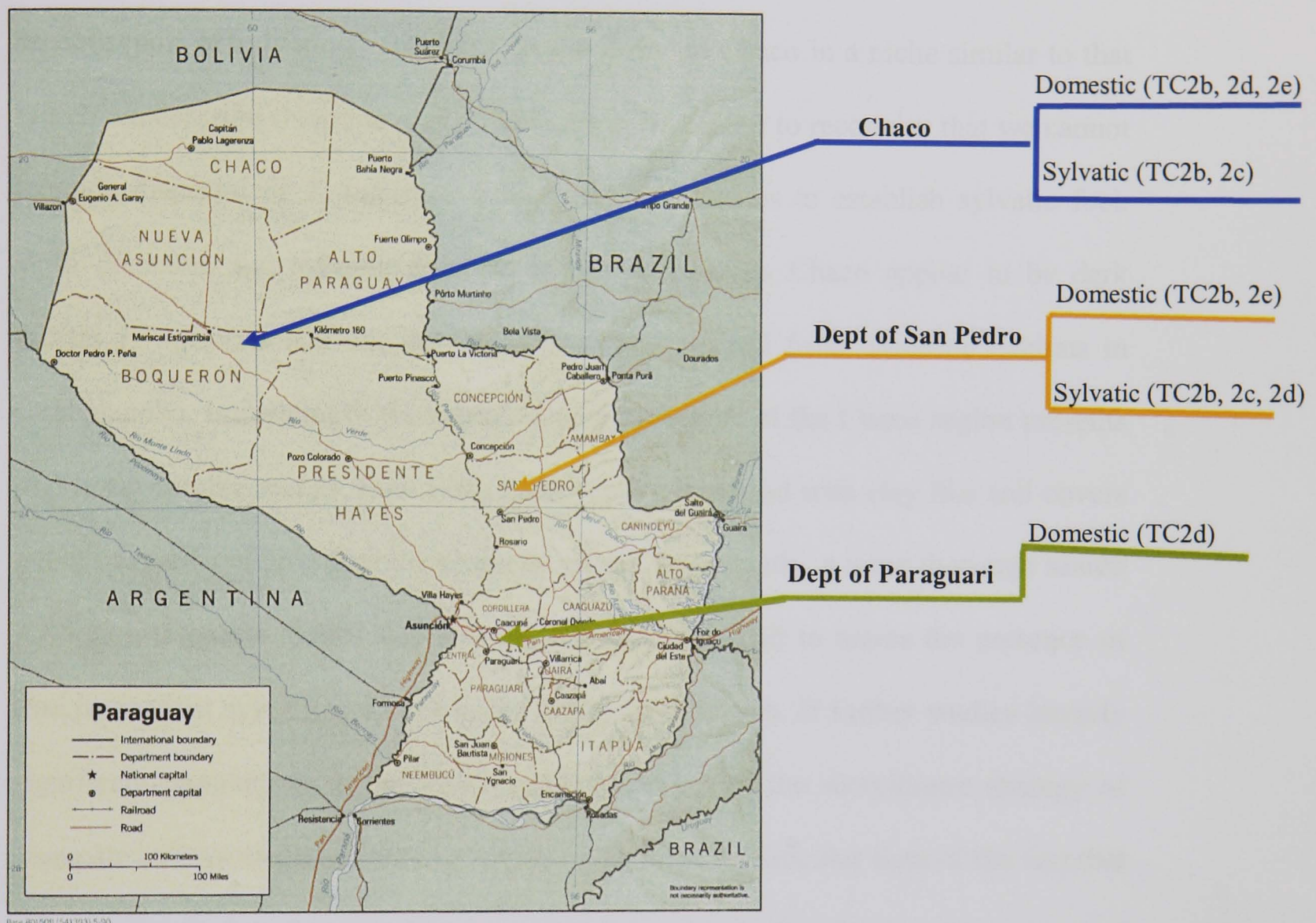


Figure 48 - Domestic and sylvatic strain characterisation from different regions of Paraguay.

## 4.7 Discussion

### 4.7.1 Sylvatic *T. infestans* in the Paraguayan Chaco

The results from this work present some intriguing observations. The identification of sylvatic colonies of *T. infestans* is a potentially important find. Two sylvatic foci were identified 300m from the nearest dwelling or peridomestic structure. Additionally the most proximal dwellings had been treated with insecticide in the previous twelve months as part of the national control programme. The

relatively large numbers of bugs captured from two locations and the presence of nymphs and adults are evidence of breeding sylvatic colonies. This is contrary to the dogma that sylvatic *T. infestans* only occurs in the Cochabamba valley of central Bolivia in association with caviomorph rodents. However it is consistent with the recent report of sylvatic *T. infestans*, in the Bolivian Chaco in a niche similar to that which we describe (Noireau *et al.*, 1997). It is important to recognise that we cannot exclude dispersal of *T. infestans* from domestic colonies to establish sylvatic foci. Both domestic and sylvatic colonies in the Paraguayan Chaco appear to be dark morph *T. infestans* and not the “light morph” described from domestic habitats in Cochabamba. Interestingly the natural ecology of much of the Chaco region presents highly favourable niches. Low lying thick scrub compacted with clay like soil covers a huge expanse of land forming a natural adobe. Importantly, a more thorough search following transects deeper into sylvatic habitats is needed to assess the presence of true permanent sylvatic colonies in the Paraguayan Chaco. If further studies identify significant populations in the Chaco region a review of the surveillance strategy of domestic control measures may be necessary. This is in particular light of the fact that sylvatic *T. infestans* from the Chaco were found to be positive for *T. cruzi* and two of these characterised as infected with sub strain TC2b which has been previously found circulating in domestic cycles (Acosta *et al.*, 2001).

Two other species of triatomine, *T. guasayana* and *T. sordida* were found together in the same niche with *T. infestans*. *T. guasayana* and *T. sordida* are widely regarded as being closely related. In fact Noireau *et al.* (1998) identified 3% putative hybrids in sylvatic specimens in the Bolivian Chaco suggesting a recent evolutionary



divergence. *T. guasayana* and *T. sordida* have eclectic feeding habits, although *T. sordida* is commonly quoted as associating with avians (Lent & Wygodzinsky, 1979). The collection of rodent droppings and amphibians and reptiles suggests a variety of food sources is available. *T. guasayana* has been previously reported in association with reptiles and amphibians (*Bufo arenarum*). Future bloodmeal analysis of samples collected from all dissected specimens should help clarify the existence of any specific host associations. The relatively high density of three species of bug in the same ecotope was unexpected when considering they must compete for resources. Previous laboratory studies give some indication that *T. infestans* out-competes *T. sordida*; *T. infestans* following a logistic growth model resulted in the extinction of *T. sordida* within one year in an artificially created ecotope using chickens as the host (Oscherov *et al.*, 2001). Despite attempts to collect sylvatic specimens from 80 standing palms no bugs could be found. However many specimens of *T. sordida* were recovered from a previously felled palm in the same collection site suggesting that *T. sordida* is predominantly terrestrial. This is quite puzzling as vast areas of standing palms exist presenting a substantial ecological niche, which is presumably accessible to birds. Additionally *T. sordida* has been identified in palms (Lent & Wygodzinsky, 1979).

Dissection of 123 sylvatic bugs (51 *T. guasayana*, 45 *T. infestans* and 27 *T. sordida*) revealed five positive specimens of *T. infestans* (isolates from two were successfully cultured *in vitro*). *T. infestans* is known to be more specific in its preference for mammals than *T. sordida*. The discovery of *T. cruzi* in *T. infestans* but not in *T. guayasana* or *T. sordida* is perhaps not surprising. It is possible that light infections were missed in the latter 2 species but 10% of bug faeces were inoculated into growth media with no positive results. A simple explanation of these data is that

sylvatic *T. infestans* are feeding on (*T. cruzi* infected) terrestrial rodents, or edentates, whereas sylvatic *T. sordida* and *T. guasayana* are feeding on amphibian, or possibly bird blood.

#### **4.7.2 Characterisation of Paraguayan *T. cruzi* strains**

A combination of small and large subunit rRNA and mini-exon PCR profiles enabled the consistent resolution of four *T. cruzi* strains, that is TC1 and 3 of the five TC2 subgroups, TC2a, TC2c and TC2d. However TC2b and TC2e could not be resolved by this approach, as both produced 18S rRNA amplification products in the 165bp region. This is at odds with Brisse *et al.* (2001) who reported resolution of these two strains with the 18S rRNA marker, but only because TC2e was identifiable by lack of amplification. Our consistent 165bp marker was double checked with cloned stocks of DNA from different sources. Resolution of TC2b and TC2e could be achieved by the use of any of the four enzymes that we applied. PCR amplification and relatively few enzymes could thus identify all 6 *T. cruzi* subspecific designations.

PGM, GPI and 6-PGD enzymes produced fixed heterozygous profiles from samples covering a wide geographical region. This at first appears consistent with clonal propagation (Tibayrenc *et al.*, 1990 ). The presence of fixed heterozygosity has been interpreted as evidence of historic recombination (Chapman *et al.*, 1984), which has recently been supported by evidence that CL Brener is in fact a hybrid of TC2b and TC2c (Ayala & Machado, 2001). Furthermore *T. cruzi* has now been demonstrated to hybridise via an unusual mechanism (Gaunt *et al.*, 2003). The Paraguayan *T. cruzi* strains in the context of genetic hybridisation will be mentioned again in chapter 5.

Remarkable strain diversity in Paraguay is revealed by our results, in agreement with the limited previous studies. We identified TC2b, TC2c, TC2d and TC2e. TC2b, TC2d and TC2e as domestic isolates and TC2b, TC2c and TC2d as sylvatic isolates; TC2c has been isolated previously in the domestic habitat although it was less abundant than TC2e (Chapman *et al.*, 1984, isolates X0, X109, X110). Importantly strains with homozygous enzyme profiles (TC2b, TC2c) are sympatric with hybrid strains (TC2d, TC2e) in Paraguay. More extensive collection of sylvatic isolates is needed to reveal if TC2e circulates sympatrically in sylvatic cycles. It is notable that TC2e predominates in domestic transmission cycles (55.2%) in confirmation of earlier data (Chapman, 1984).

Multiclonal *T. cruzi* infections in triatomine bugs have been reported but little explored, in part due to previous difficulties in obtaining clones due to preferential outgrowth during culture. Bosseno *et al.* (1996) reported evidence of mixed infections in 43% of Bolivian *T. infestans* by using a combination of PCR and clone-specific DNA hybridisation on triatomine faeces. After culture *in vitro* mixed infections were determined as just 6%. This partially explains the lack of mixed infection profiles in parental isolates yet detection of bi-clonal mixed infections. Four of 29 (13%) domestic *T. infestans* possessed mixed infections, found in both San Pedro and Paraguari. Strains TC2b and TC2e were identified. It is striking that the *T. cruzi* TC2e isoenzyme profiles overlap with those of TC2b, therefore TC2b in a TC2b/2e mixed infection could well be obscured by TC2e. Furthermore TC2e grows particularly vigorously *in vitro*, in accord with the fact that more TC2e clones were isolated from the mixed plates, and so might be expected to predominate in a mixed TC2b/2e infection. This might also be a factor in the prevalence of TC2e in domestic transmission cycles in Paraguay. A maximum of 8 clones were examined from each

bug so additional strains might be present in single vectors. Multiclinality has potentially important ramifications, for example providing an opportunity. or indicator of, genetic hybridisation. In *T. brucei* this occurs in the insect vector (Gibson, 2001). In contrast hybrids of *T. cruzi* have been obtained from the mammalian stage of the parasite life cycle (Gaunt *et al.*, 2003). It seems premature to exclude entirely the possibility of recombination in triatomine bugs. Isolation of clones from sylvatic mammals was not achieved in this study but will be attempted again in future research. Anion exchange separation (Lanham's column) will be tested as a means of concentrating blood from trypomastigotes of *T. cruzi* prior to plating (Lanham *et al.*, 1984). This method functions to perfection with *T. brucei* but is less effective with *T. cruzi* due to the smaller differential in surface charge between the red blood cells and trypanosome.

#### **4.7.3 *T. cruzi* in sylvatic mammals**

Twenty five percent of the mammals captured were infected with *T. cruzi*. Notably 61.9% of *D. novemcinctus* were infected, and from both the Chaco region and San Pedro. Of the 13 animals, 11 carried TC2c and one TC2d (one sample in Paraguay remains uncharacterised). Two specimens of *E. sexinctus* (15.8% of the total number of this species captured) were infected, one with TC2d and one with TC2b. It appears that these two species are important reservoirs for a number of strains. The range of *D. novemcinctus* extends from Peru and northern Argentina to the south-central and south-eastern United States. Interestingly it is also found on the islands of Grenada, Trinidad and Tobago. It has been associated with *P. geniculatus* and the omnivorous behaviour of this animal may contribute to the high infection rates. Burrows are often shared among mammals, such as armadillos, rodents and



opossums, which raises the possibility of transmission across animal species by association in the same niche. One likely vector candidate is *P. megistus*, a species that is also a secondary domestic vector in Paraguay and has been identified previously in armadillo burrows (Lent & Wygodzinsky, 1979), raising the possibility of a transmission link between sylvatic and domestic cycles. This vector has been found in a variety of sylvatic ecotopes including burrows, bird nests, bat caves, hollow trees and palm tree crowns. In fact *P. megistus* was the principal domestic vector of *T. cruzi* in Brazil but since the 1930s was progressively replaced in domestic habitats by *T. infestans*. Two other species of captured armadillo (*T. matacus* and *Chaetophractus* spp.) were negative for *T. cruzi*. *T. matacus* does not construct its own burrows and although insectivorous feeds mainly on termites. *Chaetophractus* spp. although constructing their own burrows are nomadic and rarely use the same burrow twice. These traits may well explain the lack of observed *T. cruzi* infections in these species. However xenodiagnosis was not performed in the latter species.

It is increasingly apparent that the relationship between vectors, hosts, and *T. cruzi* associations is a complex and not fully resolved. However these data have shown that sylvatic armadillos are a natural reservoir host of *T. cruzi* (Tc2c, TC2b and TC2d) now propagated in the domestic cycle. It is possible that TC2c is primarily a terrestrial strain in this region with a historic association with *D. novemcinctus*. It will be interesting to try and identify the sylvatic host of *T. cruzi* 2e, which is also common in the domestic cycle. No infected specimens of *D. albiventris* were captured in this work. However future captures will be of interest as it has been found with extremely high infection rates in neighbouring Brazil (Grisard, 2000).

The possibility of further subgroups of *T. cruzi* 2, with undiscovered host and vector associations cannot be excluded. Continued efforts into the resolution of

sylvatic cycles and understanding their interaction with domestic cycles remains an important omission in current knowledge.

## 5.0 GENETIC RECOMBINATON IN *T. cruzi*

The presence of genetic exchange in *T. cruzi* and *Leishmania* is a contentious issue (Gibson & Stevens, 1999). Population genetics analyses imply overwhelming clonal reproduction and consequently a lack of genetic exchange. The majority of studies have used natural isolates, from widely dispersed geographical localities, and have tested them for Mendelian genetic exchange (panmixia) by the Hardy Weinberg equilibrium test, or for departure from panmixia by linkage disequilibrium tests (Brisse *et al.*, 2000; Tibayrenc & Ayala, 2002). Despite this, there is increasing evidence of genetic exchange in *T. cruzi*. Recent phylogenetic evidence suggests that TC2d and TC2e may have an historical hybrid origin based upon multilocus sequence data (Machado & Ayala, 2001; Tibayrenc & Ayala, 2002). The situation is complicated by the inherent plasticity of the *T. cruzi* genome. McDaniel & Dvorak (1993) undertook DNA quantitation studies on biological clones from a single strain. Results demonstrated a 30-70% increase in DNA in some clones compared to the parental stock. This indicates a mechanism allowing a radical change in DNA content. The following work is a reconciliation of all these observations by the additional demonstration of genetic hybridisation in *T. cruzi*, supported by evidence that this mechanism contributes to aneuploidy observed in natural populations. The presence of aneuploidy in a wide variety of other species, including the related protozoa *Leishmania* (Cruz *et al.*, 1993) is currently of great interest in the context of the mechanisms and implications of genome duplication, particularly as an evolutionary process (Ohno, 1970; Knight, 2002).

This section of the PhD programme was a continuation of work commenced by others, and was performed in collaboration with others, as indicated in Table 4.1 and in parentheses by reference to co-authors on Gaunt *et al.* (2003).

Table 5.1 Contributions to analysis of experimentally derived *T. cruzi* hybrids.

Method	Participants
Isoenzyme analysis	Yeo; Carrasco
Karyotype analysis	Yeo; Frame; Stothard
RAPD analysis	Yeo; Carrasco
Southern analysis	Yeo
Microsatellite analysis	Gaunt*
Sequencing of nuclear targets	Yeo; Gaunt; Veasey
Sequencing mitochondrial targets	Yeo; Gaunt; Veasey
Phylogenetics analysis	Gaunt

\*Hybrid clones cultured and, DNA extracts prepared by Yeo. Taylor prepared a transformation vector; Miles designed the project and obtained the funding.

### 5.1 Previous work performed on *T. cruzi* isolates

Two primary isolates of TC1 were obtained previously from an undisturbed enzootic cycle of transmission at a site in the Brazilian Amazon Basin where putative parental and hybrid phenotypes were circulating sympatrically (Carrasco *et al.*, 1996). A pair of putative parental biological clones, P1 and P2, were transfected (Stothard *et al.*, 1999) respectively, with a modified form of pTEX carrying the hygromycin resistance gene (M. C. Taylor, unpublished) and with pTEX (Kelly, 1997), the latter carrying the neomycin resistance gene (G418). *T. cruzi* P1-*hyg* and *T. cruzi* P2-*neo* were then passaged singly or together through all life cycle stages.

The following systems were used for the co-passage of P1-*hyg* and P2-*neo* (Stothard *et al.*, 1999; Gaunt *et al.*, 2003).

1. P1-*hyg* and P2-*neo* cultured epimastigotes were mixed in equal quantity, and grown axenically *in vitro* using supplemented RPMI 1640 medium, and at



intervals after the stationary phase (21 days) were passaged into new cultures containing each drug or both drugs (hygromycin 150µg/ml; G418 120µg/ml).

2. Mammalian cell (Vero) monolayers were infected with P1-*hyg* and P2-*neo* using stationary phase mixed axenic cultures containing epimastigotes and metacyclic trypomastigotes. Trypomastigotes from pseudocysts were recovered periodically between day 7 and 28, and grown as epimastigotes in axenic culture for drug sensitivity tests.
3. Triatomine bugs were membrane fed on mouse blood containing P1-*hyg* and P2-*neo* trypomastigotes derived from Vero cell monolayers. Bugs were dissected 25 to 30 days later; *T. cruzi* was re-isolated by culture on biphasic blood agar and passaged into axenic cultures to obtain sufficient organisms for testing drug sensitivities.
4. Groups of 3 immunocompromised (SCID) mice were inoculated with a mixture of faeces from triatomine bugs carrying P1-*hyg* or P2-*neo*. Populations were subsequently retrieved from infected mice into axenic culture and placed under drug selection.
5. Biological clones of *T. cruzi* were prepared by microscopical selection of single cells and culture

## **5.2 Methods for the determination of phenotype and genotype**

DNA purification was by phenol/chloroform extraction and ethanol precipitation or by Dneasy kits (Qiagen, UK). Amplification reactions used the following conditions:

Denaturation for 5 min at 94°C, 30 cycles of 94°C (1min), 50–62°C (1 min: depending on the primer  $T_m$ ) and 72°C (1min/1000bp), followed by 10 min extension at 72°C.

DNA primers and additional primers used in subsequent characterisation are shown in Table 5.2.

Isoenzyme characterisation was performed using PGM, GPI, ASAT, G6PD, and 6GPD systems (methods, 2.8.1). RAPD analysis was performed using 13 primers (Methods, 2.9). Karyotype analysis used a Biorad CHEF Mapper with an auto algorithm for separation of 0.4 Mb to 2.2 Mb fragments, followed by Southern blotting and hybridisation with radiolabelled probes (Methods, 2.14) This was originally performed by Frame and Stothard, repeated and checked by Yeo.

Table 5.2 Primers used for PCR amplification and sequencing.

LOCUS	PRIMER NAME	SEQUENCE (5'-3')
TCP	IG1( <i>f</i> ) <sup>13</sup> IG2( <i>r</i> ) <sup>13</sup> TCP17.1( <i>s</i> ) <sup>a</sup> TCP17.2( <i>s</i> )	GGTSGACATGCTCGGTGTGC AASCTTCAGTCCGCACTCGTG GATGATGAAGGCGTGGAAAT ATCGATTTTGCACCCTCAGT
GPI.1	S01( <i>f</i> ) S02( <i>r</i> ) GPI.1( <i>s</i> ) GPI.2( <i>s</i> )	GGCATGTGAAGCTTTGAGGCCTTTTTCAG TGTAAGGGCCCAGTGAGAGCGTTCGTTGAATAG C TGTGAAGCTTTGAAGCCTTT GGTCAGGAGAGGTGAATGGA
PGM	PGM.for( <i>f</i> ) PGM.rev( <i>r</i> )	GGGCGGA ACTACTACTGTCTG GGAGGGAGTAAAAGAAAGGAAAA
TPNI AF106855	TRX1-1( <i>f</i> ) TRX1-2( <i>r</i> )	AGTAGATCTGCCACGCGTACTTGGG TTGAAGCTTTCACCGCCAGAATTGATA
COII-ND1	ND1.3A( <i>f</i> ) COII.2A( <i>r</i> ) Mito.425( <i>s</i> ) Mito.850( <i>s</i> ) Mito.950( <i>s</i> )	GCTACTARTTCACTTTCACATTC GCATAAATCCATGTAACACMCCAC ATGCCGTCTGTAATAGGTGTCA ATCCACAAATTTTGATGATATA CAAAATTTAAACAACCGAAATATA

<sup>a</sup>*f*, forward primer; *r*, reverse primer; *s*, primer used for sequencing only; all other primers, except hyg and neo, were used for both amplification and sequencing.

### 5.3 Sequencing reactions

The following DNA sequences were amplified

1. *tpn* (Wilkinson *et al.*, 2002), tryparedoxin Genbank. AF106855: 435bp.
2. *gpi* (1038bp)
3. putative *pgm* (490bp), TIGR database. <http://www.tigr.org>, accession no. TC1375, identified by similarity between human and putative *Leishmania* PGM.
4. *tcp17*, an intergenic region (758bp, including gaps) flanked by 3' *tcp17* and partial 5' *tcpgp2* (Robello *et al.*, 2000).
5. A mitochondrial locus (1078bp) spanning the maxicircle encoded genes encoding cytochrome oxidase subunit II (COII) and ND1 (Machado & Ayala, 2001).
6. *dhfr-ts* (1042bp, Machado & Ayala, 2002).

PCR products from all loci were cloned into pGEM T (Methods 2.20), with the exception of those regions where no heterozygous alleles were detectable (mtDNA of progeny; *gpi* of some isolates). For each isolate a minimum of 3 (*gpi*) and either 3 or 6 (*tcp17*) clones were sequenced on a capillary sequencer (Beckman) or an ABI 377 using manufacturer's protocols.

### 5.4 Results

Fifty vero cell cultures infected with a mixture of P1-*hyg* and P2-*neo* yielded 1 population of organisms resistant to both hygromycin and G418. Six biological clones were isolated (1C2, 1D12, 2A2, 2C1, 2D9, 2F9) all resistant to both drugs and maintained under selective pressure. No double resistant progeny were obtained from mixed axenic epimastigote cultures, or from mixed passage through triatomine bugs. Three of seven populations derived from mixed passage in SCID mice retained hygromycin resistance only, suggesting overgrowth of P1-*hyg*. Remaining

populations were sensitive to drug selection indicating a loss of episomes *in vivo* (Frame and Stothard).

The characterisation of the double drug resistant clones was undertaken using a variety of techniques. Initially DNA amplification was undertaken to confirm that the six clones contained each of the resistance genes (Figure 49)

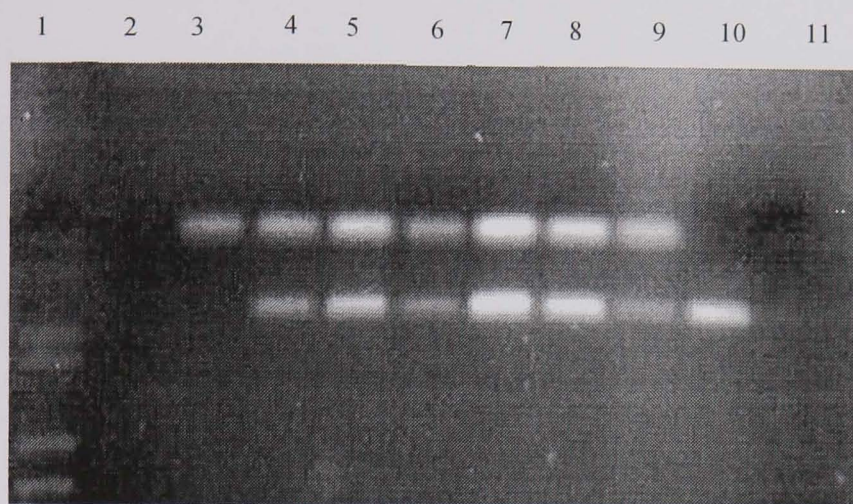


Figure 49 – Agarose gel profile from multiplex PCR products. Hybrids (lanes 4-9) contain both *hyg* and *neo* genes. Transfected parental types possess single band profiles, P1-*hyg* (lane 3) or P2-*neo* (lane 10). Order of loading from left to right. 1, 1kb Ladder. 2, P1-wild. 3, P1-*hyg*. 4-9, hybrid clones. 10, P2-*neo*. 11, P2-wild (Frame and Stothard).

#### 5.4.1 Phenotyping

Characterisation by starch gel electrophoresis was performed to identify phenotypic differences. Five enzyme systems were used, ASAT, PGM, GPI, 6GPD and G6PD. Of these PGM produced a profile which distinguished between putative parental types and hybrid clones. (Figure 50). Other systems produced identical profiles for all isolates. The enzyme system PGM clearly shows the presence of a single migrating band in each of the putative parental isolates and that the two parental types had different migration profiles. P1 possessed a slow migrating band compared that of parental type P2. Hybrids possessed both equivalents. Wild type and transfected parental stocks had the same PGM profile.



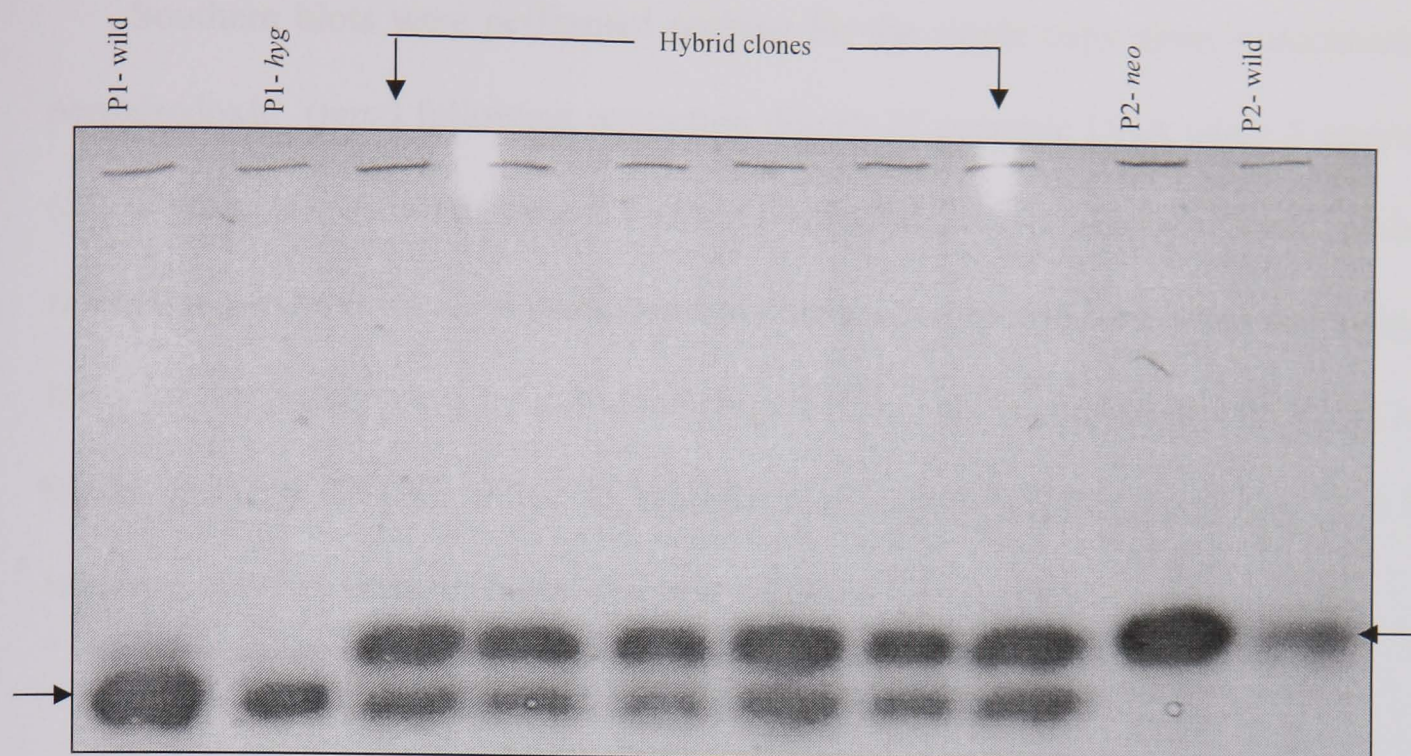


Figure 50 – Hybrids of parental phenotypes in experimentally derived double drug resistance biological clones. Starch gel electrophoresis showing PGM phenotypes (wild parental types, transformed parentals and 6 hybrid clones. Arrows indicate individual bands present in the parental types both of which are present in the hybrids (with Carrasco).

#### 5.4.2. Karyotype analysis

Further evidence of hybridisation was demonstrated with karyotype analysis of cysteine protease (*cp*) karyotype (Figure 51) showing the characteristic sharing of bands between progeny clones and parents.

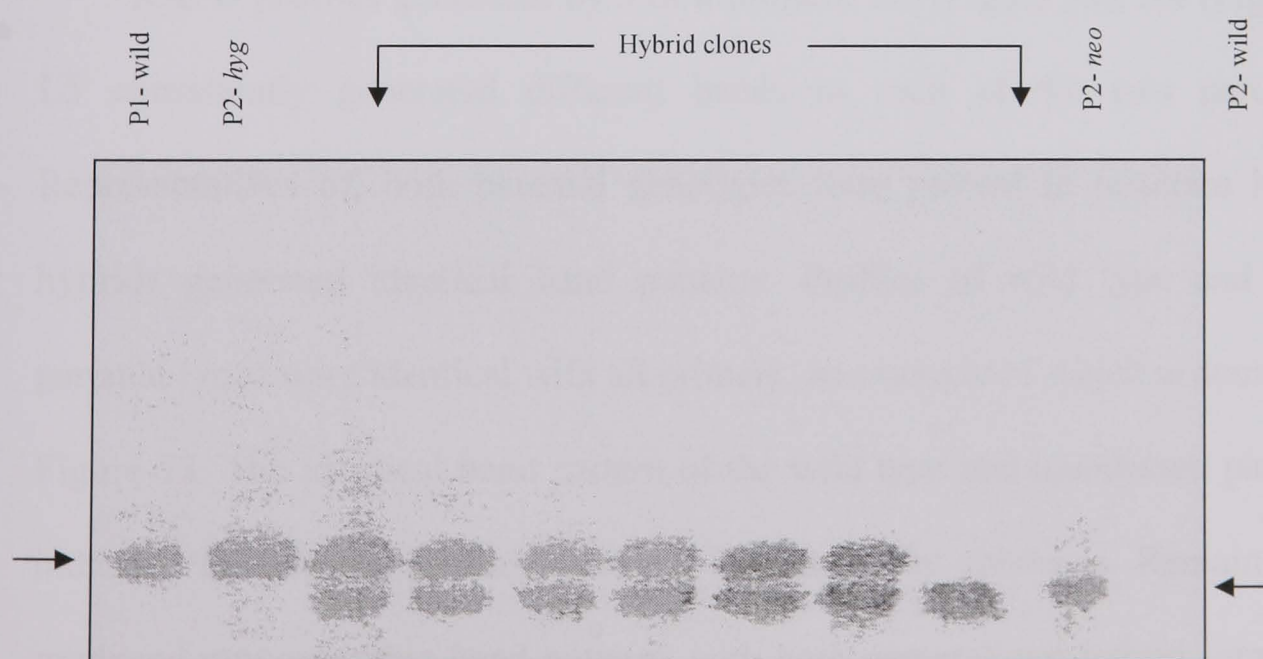


Figure 51 - Southern blot of contour clamped homogenous electric field (CHEF) electrophoresis gel probed for cysteine protease (*cp*) and showing *cp* karyotypes. Separation of 0.4 to 2.2 Mb fragments (with Frame and Stothard).



Southern blots were performed probing for the single copy gene, mitochondrial peroxiredoxin (*mpx*) following restriction digests of genomic DNA using 8 enzymes (*Sal* I, *Bam* H1, *Eco* RI, *Eco* RV, *Pst* I, *Hind* III, *Sac* I, *Xho* I). When probed, restriction products for *Xho*I produced a double banded profile for P2-*neo* and a single band for a representative hybrid clone (Figure 52). This is consistent with allele loss in the progeny. P1-*hyg* failed to produce a detectable profile suggesting that the integrity of DNA used for initial reaction was low.

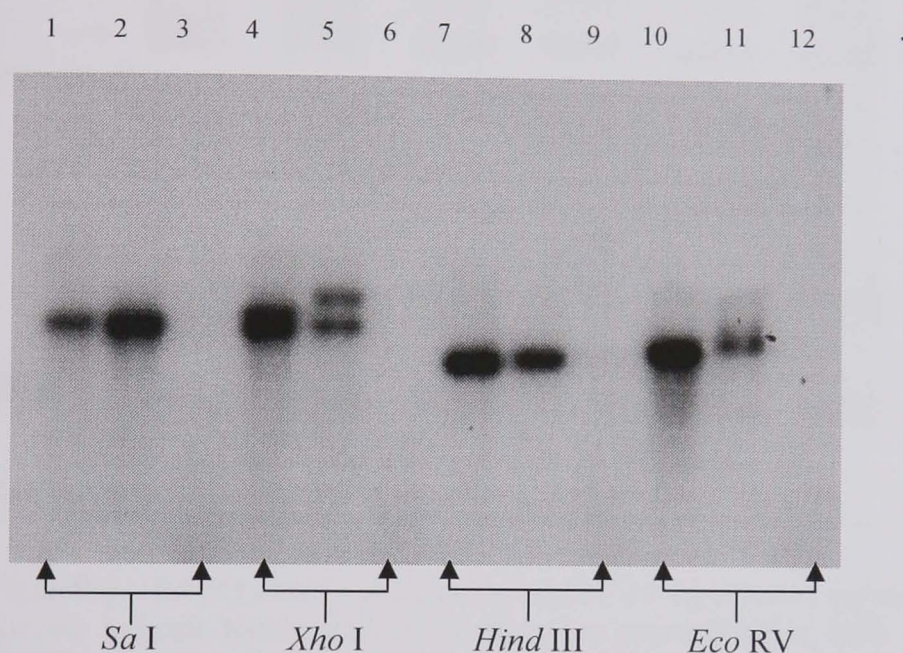


Figure 52 – Southern blot probed for *mpx* following restriction digests. Order of loading from left to right is 1, hybrid clone; 2, P2-*neo*; 3, P1-*hyg* following restriction with *Sac*I. 4, hybrid clone; 5, P2-*neo*; 6, P1-*hyg* restricted with *Xho* I. 7, hybrid clone; 8, P2-*neo*; 9, P1-*hyg* restricted with *Hind*III. 10, hybrid clone; 11, P2-*neo*; 12, P1-*hyg* restricted with *Eco* RV. No bands were detected in lanes for P1-*hyg*.

#### 5.4.3 RAPD analysis of parental types and progeny

RAPD profiles generated by 3 of 8 primers, A2 (Figure 53), A4 (Figure 54) and L5 consistently generated different bands in each of the two parental types. Representatives of, both parental genotypes were present in resultant hybrids. All hybrids generated identical band patterns. Profiles of wild type and transfected parental types were identical with all primers, an example of which is demonstrated in Figure 53. The identical band pattern of the wild type and transfected parental types indicated that hybrid profiles were not influenced by episomes. Remaining primers produced monomorphic band patterns with both parental and hybrid samples and so were not informative.



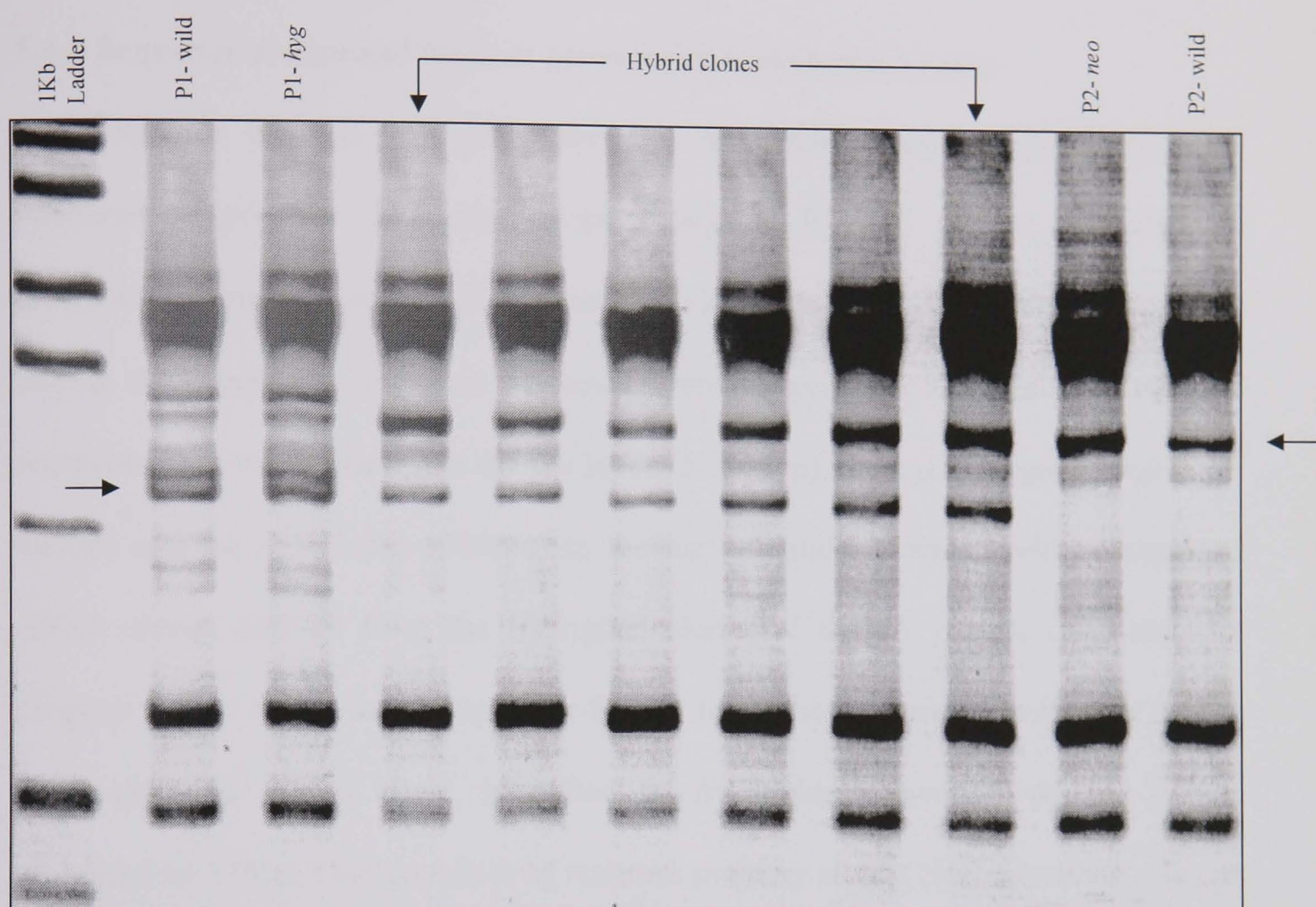


Figure 53 - RAPD profile using primer A2 showing putative parental types and corresponding hybrids. Arrows indicate bands present in respective parental types, both of which are present in the hybrids. Identical bands exist in wild type and transfected parental types.

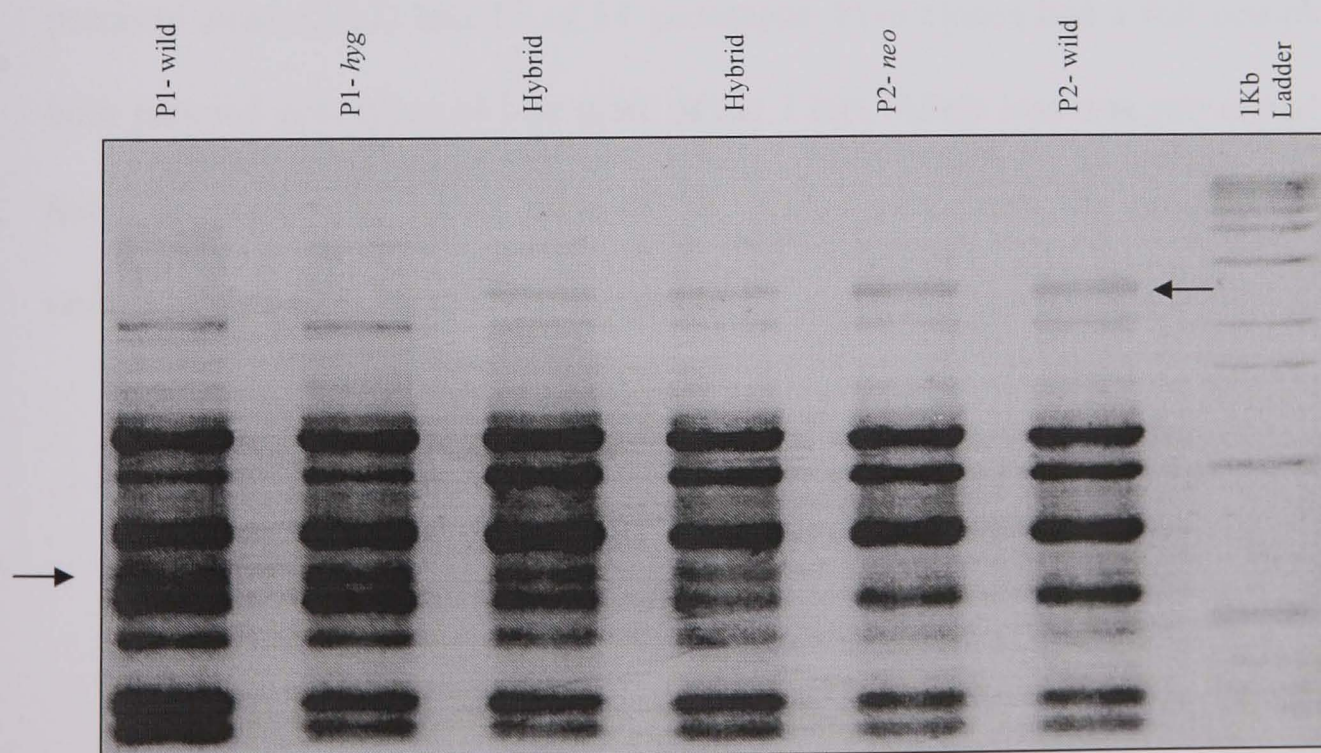


Figure 54 - RAPD profile generated using primer A4. Arrows indicate different bands present in respective parental types, both of which are present in the hybrids.

#### 5.4.4 Sequence analysis of nuclear genes (with Gaunt and Veasey)

Sequence analysis was undertaken on the nuclear genes, glucosephosphate isomerase (*gpi*), an intergenic region (*tcp17*; Robello *et al.*, 2000) and phosphoglucumutase (*pgm*). This revealed evidence of homologous recombination among the progeny in the form of mosaic parental genotypes (Table 4.4). Putative recombination was observed at the *gpi* locus (5' 600bp), the *tcp* intergenic region (5' 400bp) and the *pgm* locus (380bp), as mosaic parental genotypes within amplified cDNA clones derived from the biological clones of the six double drug resistant progeny (Table 5.3). For example, for the *gpi* locus, both parents contain one unique allele and one shared allele, identified by nucleotide polymorphisms at 2 sites separated by 276bp. One double drug resistant progeny clone (2F9) contained a fourth unique allelic combination which was a mosaic between the unique alleles in each parent. The parents P1 and P2, at the *gpi*, *pgm* and *tcp* loci had a combined total 3, 4, and 6 genotypes respectively (Table 5.3). Evidence of fusion was also apparent. One progeny clone (2A2) had 13 of 14 genotypes. Five clones had a full complement of both parental genotypes at 1 or more of the 3 loci. Allele loss was substantiated at the *tpn I* (tryparedoxin) locus: none of 30 DNA clones from the progeny carried the unique allele of the heterozygous parent.



Table 5.3 Putative recombination in cDNA clones derived from double drug resistant hybrids of *T. cruzi* (P1-*hyg* and P2-*neo*).

Locus	Genotype and nucleotide position (bp) <sup>d</sup>	No. parental clones of given genotype	No. progeny clones from 1C2, 1D12, 2A2, 2C1, 2D9 and 2F9 combined
<i>gpi</i>	939 1215		
	T T	P1- <i>hyg</i> 14	17
	C C	P2- <i>neo</i> 2	2
	T C	P1- <i>hyg</i> 16 P2- <i>neo</i> 7	39
	C   <sup>a</sup> T	P1- <i>hyg</i> & P2- <i>neo</i> 0	Putative rec.: 1C2, 2A2, 2C1, 2F9, 2D9 7

<i>tcp</i> <sup>e</sup>	1 1 2 3 3 3 1 9 0 1 4 3 4 4 1 3 9 5 5 7 9 0 1		
	T C T T A G - - - <sup>a</sup>	P1- <i>hyg</i> 3	0
	A G A A G C T T A	P2- <i>neo</i> 7	19
	W <sup>b</sup> G <sup>b</sup> T T R <sup>b</sup> G - - -	P1- <i>hyg</i> 14 P2- <i>neo</i> 17	55
	A G A A G   G - - -	P1- <i>hyg</i> & P2- <i>neo</i> 0	Putative rec.: 1C2, 1D12, 2A2, 2D9, 2F9 5
	A G T T G G   T T A	P1- <i>hyg</i> & P2- <i>neo</i> 0	Putative rec.: 2D9 1

<i>pgm</i> <sup>e</sup>	1 1 1 1 1 1 1 5 7 7 7 7 7 7 1 2 3 8 8 8 9 5 4 3 0 1 2 9		
	T G G G A A T	P1- <i>hyg</i> 2	4
	T A A G A A A	P2- <i>neo</i> 5	3
	Y <sup>c</sup> A A - - - A	P1- <i>hyg</i> 5 P2- <i>neo</i> 4	36
	T A A G A A   T	P1- <i>hyg</i> & P2- <i>neo</i> 0	Putative rec.: 2C1 1
	C   A A G A A A	P1- <i>hyg</i> & P2- <i>neo</i> 0	Putative rec.: 2A2 1
	C A A   G A A T	P1- <i>hyg</i> & P2- <i>neo</i> 0	Putative rec.: 2F9 1

rec. = recombination; | = putative site of recombination - = nucleotide gap. <sup>b</sup> 3 parental genotypes are represented by degenerate nucleotide codes (W = A or T; S = G or C; R = A or G). <sup>c</sup> 2 genotypes are represented by degenerate nucleotide codes (Y = C or T). <sup>d</sup> Position relative to *T. cruzi gpi* (Genbank accession, AC137988), or putative *Leishmania pgm* (Genbank protein accession, CAC14526). <sup>e</sup> Locus amplified using Taq Extender™ (Stratagene).



## 5.5 Mitochondrial maxicircle sequence (with Gaunt and Veasey)

Mitochondrial maxicircle sequence analysis revealed 1 differences in the NADH dehydrogenase subunit 1 (ND1) gene, between P1-*hyg* (A) and P2-*neo* (G) at positions 102bp (613bp in the amplicon sequenced). No evidence was found for split peaks in the section of the mitochondrial sequence. Five progeny (1C2, 2A2, 2C1, 2D9, 2F9) inherited the P1-*hyg* genotype and 1 (1D12) inherited the P2-*neo* genotype (Table 4.5). Parental cytochrome oxidase II regions were also sequenced. All progeny and parental profiles were identical.

Table 5.4 Mitochondrial maxicircle sequence polymorphisms (NADH dehydrogenase subunit 1 (ND1) gene.

Strain	Sequence 604-624bp
P1- <i>hyg</i>	ATAAAAA <b>CA</b> CAAAAA <b>CA</b> TA
P2- <i>neo</i>	ATAAAAA <b>CAG</b> CAAAAA <b>CA</b> TA
2A2	ATAAAAA <b>CA</b> CAAAAA <b>CA</b> TA
2D9	ATAAAAA <b>CA</b> CAAAAA <b>CA</b> TA
2F9	ATAAAAA <b>CA</b> CAAAAA <b>CA</b> TA
2C1	ATAAAAA <b>CA</b> CAAAAA <b>CA</b> TA
1C2	ATAAAAA <b>CA</b> CAAAAA <b>CA</b> TA
1D12	ATAAAAA <b>CAG</b> CAAAAA <b>CA</b> TA

## 5.6 Discussion

This work demonstrates the extant capacity for genetic exchange. The experimental observations are based on karyotype analysis (cysteine protease), Phenotyping (PGM), RAPD analysis (3 of 7 primers) and sequencing (three loci). The recombination event/events occurred in the mammalian stage of the life cycle. This appears logical as a systemic infection with multiple strains may be carried for life. This is unlike genetic recombination of the related trypanosome *T. brucei* (separated  $\geq 100$ mya) where recombination takes place in the insect vector and is believed to involve meiosis. Mitochondrial maxicircle sequence polymorphisms demonstrated uniparental inheritance patterns and is in line with established thinking, bi-parental inheritance is so far only identified in mice, mussels, *Drosophila* and humans (Kvist *et al.*, 2002).

Elements of this experimental work were performed in parallel with M. Gaunt who also demonstrated strong experimental evidence that the six experimentally produced clones are the result of a tetraploid fusion event using 10 informative microsatellite loci (Gaunt *et al.*, 2003). Allele loss was demonstrated by the disappearance of an entire microsatellite marker (L660, Gaunt *et al.*, 2003), and *tpni* sequence markers in the progeny. Southern blot analysis probing for *mpx* produced a double banded profile for P2-*neo* and a single band for a representative hybrid clone consistent with these observations of gene erosion. Mosaic genotypes also indicated recombination events.

The observations in experimentally derived recombinants were paralleled by the microsatellite polymorphisms (within biological clones) for five field isolates of *T. cruzi* (1 of TC1 and 4 of TC2). This raises the possibility that multiple microsatellite

genotypes previously taken to indicate mixed infections (multiclinality) could also be explained by polyploidy (Gaunt *et al.*, 2003). It is known that the well studied strain CL Brener diverges from uniform diploidy with the presence of triploid sections of the genome (J. Kelly, personal communication). Additionally, phylogenetic analysis demonstrated mosaics between TC2b and TC2c, and TC2b and TC1 (Gaunt and Miles, personal communication). This is remarkable as the two distinct lineages are considered genetically quite distant. Recombination in the experimental hybrids also accords with that of the reference strains and field isolates. Prior to analysis the experimental hybrids had undergone many mitotic divisions. It has been shown that high levels of mitotic recombination via gene conversion are known from other organisms, including the group of eukaryotic pathogens, the oomycetes (Chamnanpant *et al.*, 2001).

Together these results demonstrate that present and historic genetic recombination has occurred in *T. cruzi* via a proposed mechanism of fusion (and polyploid formation) followed by genome erosion and stabilisation. This stabilisation by gene erosion is possibly a mechanism to improve the viability of hybrids as in other species reduced fitness is often associated with polyploidy, resulting in reproductive isolation (Delneri *et al.*, 2003; Lynch & Conery, 2000). The implications of recombination events are potentially significant. Aneuploidy via a hybridisation mechanism explains the varying amounts of DNA found in *T. cruzi* strains and clones (McDaniel & Dvorak, 1993). Importantly this mechanism encompasses the view of apparent clonal propagation, yet occurrence of recombination. Similarly it has been known for some time that panmixia in certain bacterial species is not observed even when frequent recombination is occurring. Importantly aneuploidy via nuclear hybridisation is a non-Mendelian mechanism, and as such aneuploidy followed by



subsequent allele loss would cause the observed deviation from Mendelian heterozygosity. Mechanisms resulting in considerable exchange in genetic information may make it possible for rapid speciation and adaptation to new host environments. In flowering plants where polyploidy is common several epigenetic processes may accompany nascent allopolyploidy, such as nuclear dominance, gene silencing and mobile element activation, the latter also resulting in genetic change. Additionally in yeasts (*Saccharomyces 'sensu stricto'*), viable aneuploid hybrids have been produced (Delneri *et al.*, 2003). With this mechanism new interactions become possible as a consequence of the merger of two previously isolated diploid genomes, variously leading to intergenomic colonization and/or homogenization of formerly diverged sequences.

Although the full implications of hybridisation and aneuploidy events remain unresolved and a direct connection to adaptation remains to be established, the diversity of genetic responses to allopolyploid formation and their apparent high frequency in plants suggest that non-Mendelian phenomena contribute directly to diversification (Liu & Wendel, 2002).

## 6.0 OVERALL DISCUSSION

The overall aims of this project were to optimise a solid medium technique for isolating biological clones of *T. cruzi* from vectors and mammals, to contribute to a re-examination of the degree of genetic diversity of the major lineage *T. cruzi* II (TC2) in Paraguay, and the association with vector and mammal species; and finally to contribute to experimental studies of genetic recombination of *T. cruzi*. Comments on the major findings and the scope for future work are discussed in this concluding discussion.

### 6.1 Isolation of clones by direct plating

The optimisation of the plating method for obtaining clones now allows potential benefits for a range of applications. In this work we used reference strains that were known to have vastly different growth rates in vitro (Mondragon *et al.*, 1999). One of the primary problems associated with solid media plating is the length of time for slow growing strains to produce viable colonies. This often resulted in desiccation and in past studies little success has been achieved in their propagation. In this work we were able to isolate viable colonies for successful expansion in as little as 9 days (strain X10/1) and in selected plates, after as long as 60 days. Previously in Paraguay up to 50% of strains were lost following direct inoculation of parental isolates into growth media (LIT), which seems to be highly selective, resulting in a considerable waste of valuable stocks and effort (Acosta, personal communication). The ability to isolate a range of mixed infections allows more accurate assessment of strains circulating in specific cycles where outgrowth of more virulent strains in parental cultures would otherwise occur. Additional benefits include the ability to characterise mixed infections where one would also expect an increased likelihood of

recombinants, for example in animals experimentally infected with mixed infections. The method was essential in the further characterisation of isolates used in this study when ‘pure’ clones were required and will be prerequisite for future work using multi locus sequence typing (MLST) for population and phylogenetic studies on *T. cruzi*. Furthermore the ability to use this method without the need for a CO<sub>2</sub> supplemented atmosphere enables the technique to be applied in many laboratories in South America where limited facilities exist.

Although success was achieved in the resolution of clonal colonies and mixed infections from experimentally infected mice, little success was achieved when applying the technique to sylvatic mammals. This was primarily a result of low parasitaemia. Parasitaemia can be relatively high in some natural hosts such as *Didelphis marsupialis* with associated TC1 infection. However in others, including the armadillos used in this study, and in humans, parasitaemia can be extremely low. A solution to this problem needs to be found, particularly as it appears that recombination in *T. cruzi* occurs in the mammalian stage of the life cycle (Gaunt *et al.*, 2003). In future work we hope to overcome this in mammals by using either: immunosuppressants (such as cyclosporin A (CsA) or FK506, Potter & Chang, 1999), or anion exchange separation (Lanham’s columns) (Lanham *et al.*, 1984). In this work defibrinated rabbit blood was used to prepare nutrient plates. Using *Didelphis* blood or other appropriate host animal would presumably be more sensitive. However obtaining ‘clean’ animals may be problematic. In addition, in future we will pretreat defibrinated rabbit blood at 56°C for 30 mins to inactivate complement, which may lyse epimastigotes. Contamination of plates following inoculation from bug faeces was approximately 40%. The most common contaminants were yeasts and fungi. Prior to future plating a range of antifungal agents (including 5-fluorocytosine) should

be assessed at different concentrations after incorporation into nutrient plates to improve this statistic. Interestingly collaborators have also reported that different bug species produce different contamination results (Acosta, personal communication). *T. infestans* appears more difficult to plate without contamination than *T. sordida*. This is probably a consequence of size, physiology (for example more cutaneous hair) and variations in gut fauna. Exoskeletal contamination can be reduced by a longer period of soaking in White's solution prior to dissection, although prolonged immersion would allow the solution to enter the rectal cavity killing the trypanosomes. Thus to reduce contamination from the plating of intestinal contents a range of concentrations of antibiotics and antifungals should be evaluated.

## 6.2 Characterisation of Paraguayan isolates

Our work confirms the diversity of strains of *T. cruzi* in Paraguay. TC2b, TC2c and TC2d were isolated from sylvatic environments and TC2b and TC2e from domestic environments. TC2c has also previously been identified in the domestic cycle (Chapman *et al.*, 1984). The only subgroup so far not identified in Paraguay is TC2a.

It was apparent that the armadillo species *D. novemcinctus* and *E. sexinctus* are major reservoirs of *T. cruzi* in Paraguay. Importantly, the subgroups associated with these animals (TC2c, TC2b, TC2d) have been identified in domestic cycles. We also make the association of *D. novemcinctus* and TC2c as the majority of specimens from both the Chaco region and San Pedro were infected with this strain. However TC2d was also found in one animal. In future work more animals of more species need to be obtained to identify fully the sylvatic reservoirs and associated *T. cruzi* subgroups. The sylvatic host of TC2b is not reliably known. From our results *E. sexinctus* is a



candidate natural reservoir host of TC2b. *E. sexinctus* is widely distributed in southern and central South America.

One important omission was that lack of *D. albiventris* captured in the study locations. This marsupial is found with high infection rates in neighbouring countries. However the dry arid expanse of the Chaco may not be suitable for these reservoirs. Indigenous people in the region communicated that marsupials are a rarity. However captures should be attempted further South where conditions are more humid. *D. albiventris* and *D. albiventris* are found in open and deciduous forests and in mountainous areas, whilst *D. marsupialis* is restricted to humid, broad-leaved forests. *M. domestica* and rodents, both of which also present an important overlap between sylvatic and domestic cycles, have been little studied. In Paraguay the opportunity to address the interactions between sylvatic and domestic cycles still exist as many indigenous tribes live in small isolated communities in the Chaco region. Domestic isolates from individual villages along with sylvatic mammal and bug captures from immediate surrounding locations are feasible at several sites. The tools are now available for reliable characterisation and identification of subgroups of *T. cruzi*, to determine their host associations and the presence and of genetic hybrids. For example a future study could involve initial phenotyping and genotyping of plated sylvatic and domestic isolates and MLST of multiple clones, enabling the detection of genetic hybridisation by testing for incongruence between phylogenetic trees (bootscan analysis, breakpoint analysis and split decomposition analysis). Potential targets could include genes corresponding with enzymes used in MLEE (GPI, PGM, 6-PGD and ME), but also other genes such as trypanothione reductase (*tr*), pteridine reductase and dihydrofolate reductase (*dhfrts*), beta- propeller protein (BPP1, Kelly, personal communication) and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*).

The assessment of genetic recombination and of gene flow of *T. cruzi* between cycles is now complicated by the fact that recombination events probably involve fusion and not conventional sexual recombination (Gaunt *et al.*, 2003), which means that Mendelian models of population genetics analysis do not apply. Nevertheless, whatever the model of genetic recombination, interaction between sylvatic and domestic transmission cycles can be implied if diverse genotypes are shared across the cycles, and this can be tested, for example by Mantels statistics.

Part of the impetus for this section of the project was the prediction by Miles (Gaunt & Miles, 2000) that if *Didelphis* and *Rhodnius* were the host and vectors associated with TC1, edentates would prove to be a natural host of TC2. Strikingly, armadillos in Paraguay do indeed carry TC2. The sharing of terrestrial ecotopes (burrows) by armadillos and caviomorph rodents, is consistent with transfer of TC2 from edentates to rodents, and predicts that TC2 will be found in caviomorph rodents in Paraguay, a prediction that we hope to test in future work.

### **6.3 Vectors of *T. cruzi* in Paraguay**

Two breeding colonies of sylvatic *T. infestans* were identified in this study. Though morphometric analysis of these specimens has not yet been performed, superficially they resemble dark morphs in terms of pigmentation. Previous studies indicate that sylvatic ‘dark morphs’ from Bolivia are probably separate breeding populations from domestic bugs. However this needs clarifying, so far indicators of isoenzyme electrophoresis, chromosome banding, RAPD analysis, morphometric and crossbreeding studies show them to be well within the intraspecific range (Noireau *et al.*, 1997; Noireau, *et al.*, 2000a). Additionally what appear to be dark morph forms

from photographic records have been found in domestic cycles in Paraguay (Miles, personal communication, Figure 55).



Figure 55 – Domestic dark morph *T. infestans* from the Paraguayan Chaco. Courtesy of Peter Godfrey Fonset.

The fact that two of five adults were infected with TC2b is significant as they may pose a real threat for reinvasion of domestic environments. Further field studies are essential to quantify the true range and extent of colonisation of sylvatic ecotopes. The isolated nature of nearby communities and these sylvatic ecotopes makes the study locations ideal for gene flow studies. Geometric morphometric analyses, in conjunction with molecular genetic comparisons of mitochondrial and nuclear gene sequences, will determine the relationship between sylvatic and domestic *T. infestans*, which may have a major impact on current conception on the origins and spread of *T. infestans*. Gut contents were preserved on filter paper for all species collected (*T.*

*guasayana*, *T. sordida* and *T. infestan*) and bloodmeal analysis of these samples would provide valuable insight into the sylvatic hosts of these species in Paraguay.

#### **6.4 Characterisation of genetic hybrids of TC1**

The results produced from this thesis in conjunction with work performed by others in the research group, have described a novel mechanism of genetic recombination. That is, fusion and production of aneuploids followed by gene erosion. Importantly we have shown that the mechanism involved is both historical and extant.

Current knowledge concerning fusion and the production of polyploids is limited for fungi and parasites. In some species of plants and yeasts polyploidy is often associated with reduced fitness (Delneri *et al.*, 2003) and may explain the observed gene erosion in the hybrid samples and formation of a more stable nuclear arrangement. DNA quantitation studies (McDaniel & Dvorak, 1993) using fluorescent activated cell sorting (FACS analysis), of parental and hybrid clones could give insight into the ploidy of parents and clones. Further insights into to the extent of genetic reassortment and polyploidy could be revealed by genotyping via restriction endonuclease and karyotype analysis with a range of probes aimed at single copy genes (for example those encoding glutathione peroxidase I and II (*gpxI*, *gpxII*), mitochondrial peroxidase (*mpx*), beta-propeller protein (*bpp1*), cytosolic and mitochondrial superoxidase dismutases (*cysod*, *mtsod*) and ascorbate peroxidase (*apx*). Virulence studies of any stable polyploids could be assessed by experimental *in vivo* and *in vitro* growth. Genome erosion occurring over time, may also be detected by analysing such populations at different timepoints following sequential passage.



Further research into actual mechanism of genetic reassortment would be of great interest, for instance determination of the timing and frequency of fusion events. These aspects could be addressed with transformants producing fluorescent proteins (through episomal or integrated constructs). Interaction could then be monitored by confocal microscopy (Mortara *et al.*, 2000) revealing physical aspects of the fusion mechanism. It would also be of particular interest to create hybrid lines from cells in which the drug resistance marker has been integrated into the genome as this would facilitate the study of chromosomal inheritance. Vectors could feasibly be constructed to insert the *neo* resistance gene into the RNA locus, using published sequence data, and the *hyg* gene into the beta tubulin locus. Each parental cell line would be transformed separately with each construct so that self hybridisation as well as cross hybridisation could be performed.

We detected the presence of recombination in the mammalian stage of the life cycle (Gaunt *et al.*, 2003). Therefore it follows that the likely approach to sylvatic detection is to look in the natural hosts including armadillos and marsupials. This would be particularly applicable to Paraguay where it has been proved that it is feasible to obtain mammals of several different species, and that a variety of *T. cruzi* subgroups circulate sympatrically in domestic and sylvatic environments, increasing the likelihood of multiple infections in a single animal. The lifelong nature of infection of *T. cruzi* makes it makes the prospect of genetic recombination more likely via multiple infections. The possibility of recombination in the vector should not be ignored and future work should include plating and detection of multiple clones from individual vectors. Additionally using TC2 transformants it should be possible to recreate TC2d or TC2e hybrids from putative TC2b and TC2c or TC2b and TC2a parents by co passage through the natural host of TC2.

Together using the aforementioned battery of methods, along with MLST and phylogenetic approaches (Gaunt *et al.*, 1993) a deeper understanding of transmission patterns, host association, and population structure can be achieved. This PhD programme has made a significant contribution to research in *T. cruzi*, in developing new methods for analysing multiclonality in field isolates, in discovering that armadillos are natural reservoir hosts of TC2 and in contributing to the proof of genetic hybridisation in *T. cruzi*. It has changed fundamentally perceptions of the biology and transmission cycles of *T. cruzi*.

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# APPENDIX 1

## Growth of *T.cruzi* (reference strain X10/1) on nutrient plates in a CO<sub>2</sub> incubator

	Transfer efficiency 30 days*	Transfer efficiency 60 days*	Mean col size 30 days	Mean col size 60 days
1. RPMI underlay + saline overlay	100	50	1.5	2
2. RPMI underlay + RPMI overlay	100	60	1.5	1.5
3. DIFCO (10% whole blood) + saline overlay	100	70	2	2
4. DIFCO (10% FCS) + saline overlay	100	0	0.1	0.1
5. DIFCO (10% lysed whole blood) + saline overlay	100	40	2	2
6. DIFCO (10% lysed and centrifuged Blood) + saline overlay	100	50	2	2
7. DIFCO (10% whole blood) + RPMI overlay	100	70	2	2
8. DIFCO (10% FCS + RPMI overlay)	100	30	0.5	0.5
9. DIFCO (10% lysed blood) + RPMI overlay	100	20	2	2
10. DIFCO (10% lysed and centrifuged blood) + RPMI overlay	100	40	2	2

\* Transfer efficiency was based on the expansion of 10 colonies selected at random from each plate type into 0.5ml of supplemented RPMI.

Growth of *T.cruzi* (reference strain X10/1) on nutrient plates sealed with parafilm

	Transfer efficiency 30 days	Transfer efficiency 60 days	Mean col size 30 days	Mean col size 60 days
1. RPMI underlay + saline overlay	100	70	0.5	0.5
2. RPMI underlay + RPMI overlay	100	60	0.6	1
3. DIFCO (10% whole blood) + saline overlay	100	80	0.5	1
4. DIFCO (10% FCS) + saline overlay	100	20	0.1	0.1
5. DIFCO (10% lysed whole blood) + saline overlay	100	10	1.5	1.5
6. DIFCO (10% lysed and centrifuged Blood) + saline overlay	100	60	2.2	2.2
7. DIFCO (10% whole blood) + RPMI overlay	100	70	1	2
8. DIFCO (10% FCS + RPMI overlay)	100	0	0.5	0.5
9. DIFCO (10% lysed blood) + RPMI overlay	100	0	0.5	1
10. DIFCO (10% lysed and centrifuged blood) + RPMI overlay	100	0	0.5	0.5



Growth of *T.cruzi* (reference strain Esm cl2) on nutrient plates incubated in a 4% CO<sub>2</sub> incubator

	Transfer efficiency 30 days	Transfer efficiency 60 days	Mean col size 30 days	Mean col size 60 days
1. RPMI underlay + saline overlay	100	80	1	1
2. RPMI underlay + RPMI overlay	100	60	0.5	0.5
3. DIFCO (10% whole blood) + saline overlay	100	80	0.1	0.5
4. DIFCO (10% FCS) + saline overlay	0	0	0	0
5. DIFCO (10% lysed whole blood) + saline overlay	100	0	1	1
6. DIFCO (10% lysed and centrifuged Blood) + saline overlay	100	0	1	1
7. DIFCO (10% whole blood) + RPMI overlay	100	0	0.5	0.5
8. DIFCO (10% FCS + RPMI overlay)	0	0	0	0
9. DIFCO (10% lysed blood) + RPMI overlay	100	30	1.5	1.5
10. DIFCO (10% lysed and centrifuged blood) + RPMI overlay	100	60	0.5	0.5

Growth of *T.cruzi* (reference strain Esm cl2) on nutrient plates sealed with parafilm.

	Transfer efficiency 30 days	Transfer efficiency 60 days	Mean col size 30 days	Mean col size 60 days
1. RPMI underlay + saline overlay	100	90	0.05	1
2. RPMI underlay + RPMI overlay	100	60	1	1
3. DIFCO (10% whole blood) + saline overlay	100	70	0.5	0.5
4. DIFCO (10% FCS) + saline overlay	0	0	0	0
5. DIFCO (10% lysed whole blood) + saline overlay	100	10	0.5	0.5
6. DIFCO (10% lysed and centrifuged Blood) + saline overlay	0	0	0	0
7. DIFCO (10% whole blood) + RPMI overlay	100	80	0.5	0.5
8. DIFCO (10% FCS + RPMI overlay)	0	0	0	0
9. DIFCO (10% lysed blood) + RPMI overlay	100	60	0	0.5
10. DIFCO (10% lysed and centrifuged blood) + RPMI overlay	100	20	0	0.5

Growth of *T.cruzi* (reference strain CANIII) on nutrient plates incubated in a 4% CO<sub>2</sub> incubator

	Transfer efficiency 30 days	Transfer efficiency 60 days	Mean col size 30 days	Mean col size 60 days
1. RPMI underlay + saline overlay	0	40	0.1	1
2. RPMI underlay + RPMI overlay	100	60	0.1	0.1
3. DIFCO (10% whole blood) + saline overlay	0	70	0.1	0.1
4. DIFCO (10% FCS) + saline overlay	0	0	0	0
5. DIFCO (10% lysed whole blood) + saline overlay	0	0	0	0
6. DIFCO (10% lysed and centrifuged Blood) + saline overlay	0	0	0	0
7. DIFCO (10% whole blood) + RPMI overlay	100	70	0.1	0.1
8. DIFCO (10% FCS + RPMI overlay)	0	0	0	0
9. DIFCO (10% lysed blood) + RPMI overlay	0	0	0	0
10. DIFCO (10% lysed and centrifuged blood) + RPMI overlay	100	100	0.1	0.1



Growth of *T.cruzi* (reference strain CANIII) on nutrient plates sealed with parafilm

	Transfer efficiency 30 days	Transfer efficiency 60 days	Mean col size 30 days	Mean col size 60 days
1. RPMI underlay + saline overlay	0	40	0.1	1
2. RPMI underlay + RPMI overlay	0	60	0.5	0.5
3. DIFCO (10% whole blood) + saline overlay	0	90	0.5	0.5
4. DIFCO (10% FCS) + saline overlay	0	0	0	0
5. DIFCO (10% lysed whole blood) + saline overlay	0	0	0	0
6. DIFCO (10% lysed and centrifuged Blood) + saline overlay	0	0	0	0
7. DIFCO (10% whole blood) + RPMI overlay	0	50	0.1	0.5
8. DIFCO (10% FCS + RPMI overlay)	0	0	0	0
9. DIFCO (10% lysed blood) + RPMI overlay	0	0	0	0
10. DIFCO (10% lysed and centrifuged blood) + RPMI overlay	0	0	0	0



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